



Laboratory Diagnosis of Lyme Borreliosis

 John A. Branda,^a Allen C. Steere^b

^aDepartment of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

^bDepartment of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA

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Address correspondence to John A. Branda, branda.john@mgh.harvard.edu.

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SUMMARY Lyme borreliosis is caused by a growing list of related, yet distinct, spirochetes with complex biology and sophisticated immune evasion mechanisms. It may result in a range of clinical manifestations involving different organ systems, and can lead to persistent sequelae in a subset of cases. The pathogenesis of Lyme borreliosis is incompletely understood, and laboratory diagnosis, the focus of this review, requires considerable understanding to interpret the results correctly. Direct detection of the infectious agent is usually not possible or practical, necessitating a continued reliance on serologic testing. Still, some important advances have been made in the area of diagnostics, and there are many promising ideas for future assay development. This review summarizes the state of the art in laboratory diagnostics for Lyme borreliosis, provides guidance in test selection and interpretation, and highlights future directions.

KEYWORDS *Borrelia*, *Borrelia burgdorferi*, *Borrelia*, borreliosis, diagnosis, diagnostics, Lyme disease, molecular methods, serology

INTRODUCTION

Lyme borreliosis, also known as Lyme disease, is a bacterial infection caused by spirochetes of the *Borrelia burgdorferi sensu lato* complex (also more recently grouped under a new genus taxon named *Borrelia*) (1). In this review, we retain the former taxonomy of *Borrelia burgdorferi sensu lato* complex (Bbsl), because the new taxonomy is still not widely accepted (2, 3). The causative agents of Lyme borreliosis are transmitted to humans by ixodid (hard) ticks. The infection usually begins with an expanding skin lesion, termed erythema migrans. Untreated patients may develop acute Lyme neuroborreliosis or Lyme carditis within weeks of infection, or Lyme arthritis within months of infection, with rarer manifestations also described (4). Direct detection of the infectious agent is often not possible or practical, necessitating reliance on serologic testing for support of the diagnosis. Serologic testing is insensitive during the first weeks of infection, but after that time the standard two-tiered approach of enzyme immunoassay (EIA) followed by immunoblots, or newer two-test approaches, have high sensitivity and specificity. Thus, using validated interpretative criteria, laboratory testing substantially improves overall diagnostic accuracy.

AGENTS OF INFECTION

Spirochetes of the family *Borreliaceae* are separated into two distinct phylogenetic groups: (i) Lyme-related borreliae and genetically similar species and (ii) relapsing fever borreliae and their relatives (1, 5, 6). The *Borrelia burgdorferi sensu lato* genospecies complex (Bbsl) includes the three most frequent agents of Lyme borreliosis worldwide—*Borrelia burgdorferi (sensu stricto)*, *Borrelia afzelii*, and *Borrelia garinii*—along with closely related *Borrelia* species that rarely, if ever, cause human infection (7, 8). Nearly all Lyme borreliosis cases acquired in North America are caused by *B. burgdorferi*, with the remaining few cases caused by *B. mayonii*, a recently recognized species in the upper Midwest (7, 9, 10). The *B. burgdorferi* type strain is B31, which was the original isolate, recovered from ticks collected on Shelter Island, New York (11). In Europe, most Lyme borreliosis cases are caused by *B. afzelii* or *B. garinii*, with a lesser contribution from *B. burgdorferi*, *B. spielmanii*, *B. bavariensis*, and rarer species (8). In Asia, *B. garinii* predominates as the causative species (8).

Each of the three most important pathogenic species is associated with certain differences in clinical expression. For example, classic Lyme neuroborreliosis (Bannwarth's syndrome) in Europe is associated with *B. garinii*, whereas *B. afzelii* more typically causes skin manifestations (12–16). *B. burgdorferi* in the northeastern and mid-Atlantic United States is particularly arthritogenic, accounting for the greater frequency of Lyme arthritis cases in North America compared to Europe or Asia (8, 16, 17). Regional differences in species distribution can have important implications for diagnostic testing as well, since diagnostic assays intended to detect infection by one species may

perform less well in detecting infection by another species (18–22). This is less of a problem in the United States, where almost all domestically acquired infections are caused by *B. burgdorferi*.

Intraspecies diversity allows subclassification of clinical strains. Several phenotypic and genetic strain-typing systems have been used to separate clinical strains of a given species into subgroups (5, 16). In North America, a common strategy to differentiate among *B. burgdorferi* strains has been analysis of a single genetic locus, either the plasmid-located outer surface protein C gene (*ospC*), or the chromosomal 16S–23S rRNA intergenic spacer (IGS) region (5, 23). *ospC* sequence analysis divides North American *B. burgdorferi* strains into at least 23 *ospC* genotypes (24–27), whereas restriction fragment-length polymorphism analysis of the 16S–23S rRNA intergenic spacer divides *B. burgdorferi* strains into 3 major groups, ribosomal spacer type 1 (RST1) through RST3 (28, 29). A third system, multilocus sequence typing, which is based on sequence analysis of 8 housekeeping genes, divides *B. burgdorferi* strains into at least 33 sequence types (30). Notably, *B. burgdorferi* populations in Europe and North America constitute distinct lineages (30), and clinical isolates collected from patients in the Northeast or Upper Midwest regions of the U.S. represent distinct *B. burgdorferi* populations (27).

The existence of divergent genotypes within *Borrelia* species has clinical and diagnostic implications. First, exposure to one genotype does not necessarily confer immunity to other genotypes (31), and serial distinct infections caused by *B. burgdorferi* strains of different genotypes are possible in the same individual (32–35). Reinfection may occur after an episode of antibiotic-treated erythema migrans (36, 37), whereas reinfection is very rarely documented after resolution of a late Lyme borreliosis manifestation, presumably because the expanded immune response associated with the latter is more broadly protective (37).

Second, in Europe, where there is greater diversity of genospecies, optimization of serologic assays requires inclusion of antigens or epitopes derived from the prevalent genospecies (38–42). To a lesser degree, genotypic diversity among North American *B. burgdorferi* strains may also have the potential to affect serologic test performance. For example, antigenic differences linked to genotype might explain (at least in part) why the sensitivity of immunoblots prepared from the original isolate of *B. burgdorferi* (strain B31, an RST1 isolate which is used in most North American serologic assays) is higher in patients infected with RST1 strains than in patients infected with RST2 or RST3 strains (42).

Third, some *B. burgdorferi* genotypes are more virulent than others (27). For example, the *B. burgdorferi* RST1 subtype has greater inflammatory potential (43, 44), is more frequently detectable in blood (23, 45, 46), and is associated with more severe early disease and with higher rates of postinfectious, antibiotic-refractory Lyme arthritis (44, 47). In the Northeastern United States, more than half of isolates from EM skin lesions are *OspC* type A (part of the RST1 group) or *OspC* type K (part of the RST2 group), whereas in the upper Midwest, *OspC* type H (also in the RST2 group) appears to be most common (27). Certain *OspC* genotypes, especially types A, B, H, I, and K, confer a higher risk of dissemination (23, 26, 27, 46, 48). Thus, genotype-associated virulence factors, combined with certain host factors, account (at least in part) for the wide variation in clinical manifestations and outcomes among patients with Lyme borreliosis (44).

TRANSMISSION, EPIDEMIOLOGY, AND RISK FACTORS

Agents of Lyme borreliosis are transmitted between reservoir hosts and humans (incidental hosts) by hard-bodied ticks of the *Ixodes ricinus* complex (49). *B. burgdorferi* must be coated with a tick protein, Salp15, to survive initial transmission from tick to host (50). Neither direct person-to-person transmission, direct zoonotic transmission, nor transmission via blood product transfusion has ever been documented, although Lyme-related borreliae can survive in packed red blood cells at refrigeration temperatures and transfusion-acquired infection is a theoretical transfusion risk (51, 52).

Vertical human transmission leading to congenital infection has also not been described (53–56). Most infections are acquired from nymphal ticks rather than adult ticks (57), in part because transmission usually requires 36 to 48 h of tick attachment, and adult, engorged ticks are more likely to be noticed and removed in that time frame compared to feeding nymphs (7, 58). Larval ticks, which are not infected, do not transmit Lyme disease.

Nymphal ixodid ticks are most active and abundant in the late spring and early summer months; this, along with greater human outdoor activity during warmer weather, accounts for the marked seasonality of erythema migrans cases, which peak during the summer months in the United States (59, 60). Certain later manifestations, especially Lyme arthritis, typically manifest after months of latent infection, and therefore the onset of arthritis does not follow a seasonal pattern (61). In the United States, Lyme borreliosis has a slight male predominance and a bimodal age distribution, with peak incidence at 5 to 15 years and at >45 years of age (7, 17). Risk factors include peridomestic, recreational, or occupational exposure to ixodid tick habitat (62–65).

The necessity of vector intermediates limits human transmission to specific geographical regions, in which the natural Bbsl enzootic cycle can be maintained. Areas of endemicity exist throughout temperate regions of the Northern Hemisphere, including large swaths of North America, Europe and Asia (8). In North America, most cases are acquired in the Northeast, Mid-Atlantic, and Upper Midwestern regions of the U.S. extending into Canada, and there are now highly affected areas in southern Canada, especially in Nova Scotia (66–68). However, the disease incidence is low in the U.S. Pacific Northwest, and there is minimal or negligible incidence elsewhere in the United States. (7, 32, 69, 70). Levels of endemicity can vary significantly at the local level, with pockets of hyperendemicity neighboring areas of low-disease incidence (71). Regions of endemicity are expanding (68, 71–73), mirroring territorial expansion of competent vectors (74), and the annual incidence of reported infection has increased sharply over the past few decades in North America, Europe, and Asia (7, 69).

CLINICAL MANIFESTATIONS

The most common presenting manifestation of Lyme borreliosis is an annular, expanding, erythematous skin rash termed erythema migrans (EM), which results from localized infection at the inoculation site after an incubation period of about 1 week (range, 3 to 32 days) after a tick bite (4, 75, 76). As the lesion expands, it may develop partial central clearing surrounded by a redder outer border, or classically, a “bull’s eye” configuration with concentric rings of erythema alternating with partial clearing (77). However, if the patient is seen within days of rash onset, less distinctive lesions are more common. Typical appearances include expanding, homogeneous erythema, or expanding pale erythema surrounding a darker red center (78, 79). In the United States, many patients with EM also have systemic symptoms, most frequently fatigue, arthralgia, myalgia or headache, and regional lymphadenopathy may be present (77, 80, 81). During the first days of infection, fever and chills may also be present, particularly in children. In some cases, multiple EM lesions occur (81), with a primary lesion at the site of the tick bite and secondary skin lesions resulting from hematogenous dissemination (82). Peripheral leukocytosis, leukopenia, anemia, or thrombocytopenia are not associated with EM, whereas lymphopenia or elevated liver function tests sometimes are (83).

Dissemination may also lead to noncutaneous organ involvement. If the patient is not treated with antimicrobial therapy, early Lyme neuroborreliosis may develop in approximately 15% of patients within weeks after initial infection, usually manifesting with cranial neuropathy (most commonly unilateral or bilateral facial nerve palsy), lymphocytic meningitis, or radiculitis (76, 84). Within the same time frame, a less frequent (but potentially fatal) manifestation of early disseminated disease is Lyme carditis, usually causing atrioventricular conduction block in about 5% of untreated patients (4, 84, 85). In the northeastern United States, Lyme arthritis is the most common late

manifestation of the disease, which occurs in approximately 60% of untreated patients, usually beginning months to as long as 2 years after untreated initial infection (4, 76, 86). Lyme arthritis typically affects only one or a few large joints, especially the knee (84, 86). Affected knees usually have large joint effusions, with a neutrophilic leukocytosis in synovial fluid (87). Arthritis occurs only in a small proportion of European patients with Lyme borreliosis, often earlier in the disease course than in U.S. patients (17). In contrast, several later manifestations recognized with some frequency in Europe are rare or absent among North American cases, including acrodermatitis chronica atrophicans, borrelial lymphocytoma, and late encephalomyelitis (8, 84).

In clinical practice today, most patients present with—and are treated for—erythema migrans, preventing later manifestations of the disease. Among cases reported to the Centers for Disease Control and Prevention (CDC) in recent years, 72% had EM, 28% had arthritis, 13% had neurologic involvement, and only 1.5% had carditis (88). In addition, among participants in a Lyme disease vaccine trial in the United States, 11% had asymptomatic infection (89). In Europe, as many as half of cases may be asymptomatic (90).

DIAGNOSTIC TESTING

There are two broad categories of diagnostic tests for Lyme borreliosis: (i) direct detection methods, which detect the agent of infection in primary patient specimens, and (ii) indirect detection methods, which detect a host response to the infection. The first tests adopted for routine clinical diagnostic use were serum antibody tests (91). These indirect detection assays have evolved substantially in their methodology and chemistry (92), and they remain the most useful and widely available diagnostic aids. Despite prodigious recent advances in molecular methods for direct detection of other infectious agents, these and other direct detection strategies currently play little role in clinical diagnostics for Lyme borreliosis.

Antibody Detection

Kinetics and evolution of the antibody response in untreated patients. A specific antibody response against *B. burgdorferi* is not detectable during a “window period” of several days to a few weeks after initial infection (93–95). As the antibody response develops, IgM-class antibodies directed against a relatively limited repertoire of immunogenic antigens are produced, often with rapid IgM-to-IgG isotype switching. Immunodominant early antigens include outer surface protein C (OspC; molecular weight, 21 to 25 kDa) and variable major protein (VMP)-like sequence, expressed (VlsE; molecular weight, 34 to 35 kDa), both of which are surface-exposed, outer membrane lipoproteins, and the 41-kDa flagellar protein (p41/flagellin/FlaB) (93–105). Other immunogenic antigens which may provoke an antibody response during early infection include the 37-kDa flagellar protein (FlaA), decorin binding proteins (Dbp) A and B, RevA, p66, BBK07, BBK32, BBG33, LA7, BmpA (molecular weight, 39 kDa), Flil, and several oligopeptide permeases (OppA1, -A2, and -A4) (95, 98, 102, 106–117). The sequence in which antibodies against specific early antigens become detectable, and the number of antibody specificities detectable at a given time point varies from patient to patient.

After 1 to 2 months of untreated active infection, an expanded IgG antibody response is reliably present, with reactivity against a range of immunogenic antigens, typically including most or all those specified in the IgG immunoblot interpretive criteria (Table 1), plus VlsE and potentially many others (94, 98). Lyme arthritis patients in the United States typically have an exceptionally expanded IgG antibody response to spirochetal proteins. In a microarray analysis of >1,200 spirochetal proteins, these patients had reactivity with as many as 89 antigens, particularly outer surface proteins (108). This commonly includes IgG reactivity with OspC (118), as well as spirochetal proteins that are usually expressed only in the tick, including OspA, OspD, and *Borrelia* iron- and copper-binding protein (BicA) (119). These tick program responses are only found in

TABLE 1 Interpretive criteria for North American immunoblots^a

Immunoblot	Criteria for a positive result ^b
IgM	Two of the following three bands must be present: 23 kDa (OspC), 39 kDa (BmpA), and 41 kDa (Fla)
IgG	Five of the following ten bands must be present: 18 kDa, 21 kDa (OspC), 28 kDa, 30 kDa, 39 kDa (BmpA), 41 kDa (Fla), 45 kDa, 58 kDa (not GroEL), 66 kDa, and 93 kDa

^aAdapted from reference 135.^bThe apparent molecular mass of OspC is dependent on the strain of *B. burgdorferi* being tested. The 23- and 21-kDa proteins referred to are the same.

U.S. Lyme arthritis patients, presumably because of the highly inflammatory milieu in their inflamed joints (120).

While the IgM antibody response usually wanes and may become undetectable in late active disease (91, 121, 122), the IgG antibody response persists (91, 123). However, in some cases, the IgM antibody response also persists (91, 122), and therefore the presence of specific IgM antibodies does not necessarily indicate an infection of short duration or early-stage infection.

Kinetics and evolution of the antibody response after prompt antimicrobial therapy. The early antibody response can be impacted by effective antimicrobial therapy (Fig. 1). In general, the eventual degree of expansion and maturation of the antibody response relates proportionately to the duration and extent of infection prior to initiation of effective therapy. When antimicrobial therapy is administered during the window period of seronegativity soon after initial infection, up to half of patients with localized cutaneous infection may remain seronegative during convalescence (78, 91, 93, 95, 124, 125). Failure to develop a detectable antibody response in convalescence strongly suggests that antimicrobial treatment, combined with the innate immune response, resulted in spirochetal elimination in the skin. More commonly, spirochetal killing during localized infection of short duration may dampen, rather than abrogate, the developing antibody response. Peak antibody titers may be lower and antibodies may develop against fewer spirochetal antigens compared with cases not treated promptly or in cases of disseminated Lyme borreliosis; isotype switching from IgM- to IgG-class antibodies may also be prevented for some or all antibody specificities (91, 95, 126).

Within a few weeks after successful antimicrobial treatment of early infection, or within weeks to months after antimicrobial treatment of late infection, antibody titers begin to decline, as determined by semiquantitative or quantitative EIAs. In contrast,

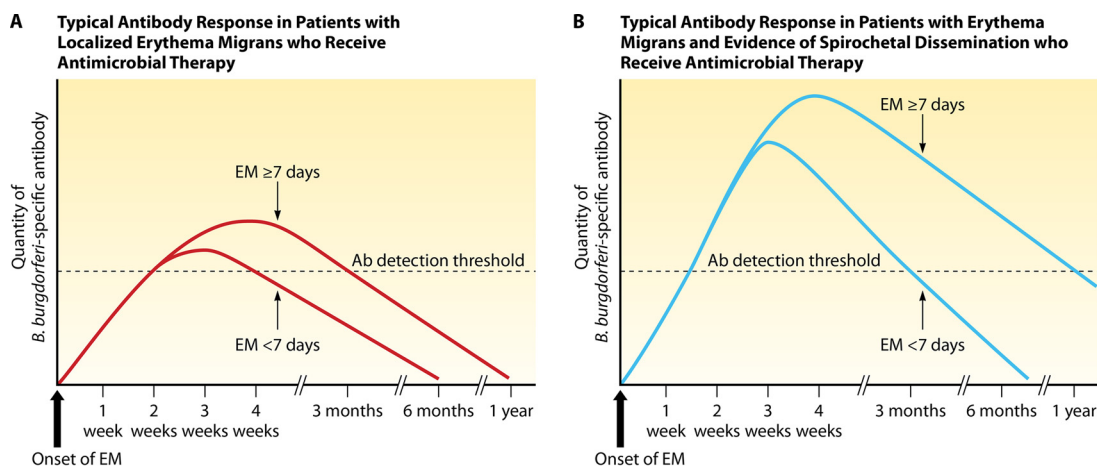


FIG 1 Kinetics of the antibody response in patients treated with antimicrobial therapy for localized erythema migrans (A) or erythema migrans with evidence of dissemination (B), as detected by polyvalent EIAs. As shown, the antibody response typically reaches a higher titer and declines less rapidly when the duration of EM prior to administration of effective antimicrobial therapy is ≥ 7 days, compared with < 7 days. (Based on data from references 93 and 95.)

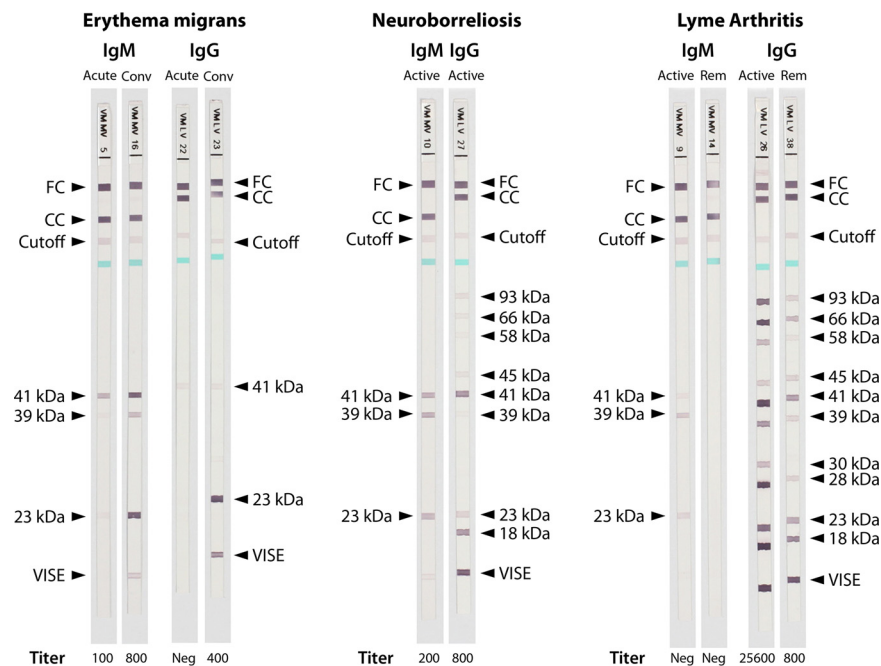


FIG 2 Ladder immunoblots demonstrating typical IgM and IgG antibody responses in individual patients with common manifestations of Lyme borreliosis. The correlating antibody titer, as measured using a semiquantitative whole-cell sonicate ELISA method, is shown at the bottom of each test strip. For the patient with erythema migrans, serum studies are shown using a sample collected during the acute phase of illness (at the time of initial presentation, prior to antimicrobial administration; strips 5 and 22) and using a sample collected in convalescence (28 days after initial presentation, following a standard course of antimicrobial therapy; strips 16 and 23). For the patient with neuroborreliosis, serum studies are shown using a sample collected during active infection (at the time of initial presentation, prior to antimicrobial administration; strips 10 and 27). For the patient with Lyme arthritis, serum studies are shown using a sample collected during active infection (at the time of initial presentation, prior to antimicrobial administration; strips 9 and 26) and using a sample collected 30 years after antimicrobial therapy (remote past infection; strips 14 and 38). Abbreviations: Conv, convalescent; Rem, remote; FC, functional control; CC, conjugate control; Cutoff, cutoff control; kDa, kilodalton; Neg, negative.

immunoblots, which are qualitative tests, do not change much, or change very slowly, after antimicrobial therapy (Fig. 2). In patients with early Lyme borreliosis, prompt treatment may result in disappearance of *B. burgdorferi*-specific antibodies over time (seroreversion) (122, 125, 127, 128), or a ≥ 4 -fold decline in IgG antibody titer (123, 129). Although antibody responses decline after effective treatment of late-stage Lyme borreliosis (122), a significant decline in IgG antibody titer is generally delayed in proportion to the duration and extent of active infection prior to therapy (93) and may require months after effective treatment of late-stage infection (129, 130). In these patients, the IgG antibody response usually remains detectable at a low level for many years, which is a sign of immune memory rather than active infection.

IgM antibodies to *B. burgdorferi* may also persist for many years after successful treatment (122), so the presence of specific IgM antibodies does not necessarily indicate active or recent infection, or reinfection, unless the appropriate clinical presentation is present. For example, in one study, 10% of patients with antimicrobial-treated early Lyme borreliosis and 15% of those with treated Lyme arthritis still had IgM seroreactivity using two-tiered testing 10 to 20 years after active infection (122).

Because *B. burgdorferi*-specific IgM and IgG antibody responses may persist qualitatively after effective antimicrobial treatment, serologic testing cannot be used to distinguish between active and past (effectively treated) infection, unless seroreversion or a ≥ 4 -fold decline in IgG antibody titer can be demonstrated by analyzing multiple serum samples collected longitudinally. Changes in antibody titer over time are best

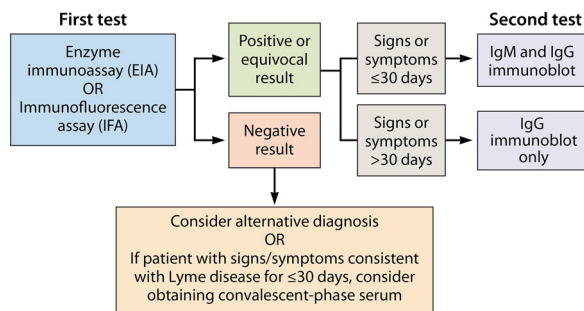


FIG 3 Conventional two-tiered serologic testing protocol for the diagnosis of Lyme borreliosis. (Adapted from reference 228.)

demonstrated using semiquantitative or quantitative EIAs. For accurate comparison, serially collected samples should be tested together at the same time, using the same assay; however, for the most part, frozen serially collected samples are only available in research settings. In clinical practice, earlier samples are usually not retained, sometimes prompting comparison of past and current laboratory reports, which is unreliable. Thus, if performed correctly, serial measurements of antibody titers can aid in monitoring the response to therapy but may be misleading if values from laboratory reports are compared from different assays or different assay runs.

If patients with a history of antimicrobial-treated erythema migrans are subsequently reinfected, as manifested by a new episode of erythema migrans, IgM-to-IgG isotype switching occurs more rapidly compared to early infections in naive hosts (131–133). IgM bands present on immunoblots during previous episodes remain and may intensify, but IgM antibodies against additional spirochetal antigens typically do not develop (35). Instead, additional IgG bands may sometimes appear early in the course of infection, indicating a more rapid expansion of the IgG antibody response than is typically associated with primary infection (35).

Conventional two-tiered serologic testing. Current U.S. guidelines for the performance and interpretation of serologic tests for Lyme borreliosis were adopted in 1994, at the Second National Conference on Serologic Diagnosis of Lyme Borreliosis in Dearborn, Michigan, with support from the CDC, the U.S. Food and Drug Administration (FDA), the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD), the National Institutes of Health (NIH), the Council of State and Territorial Epidemiologists, and the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) (134). These guidelines established and standardized a two-tiered testing protocol (Fig. 3), which was intended to maximize clinical sensitivity in the first tier, and to maximize specificity in the second tier. This has become known as conventional or standard two-tiered testing. In the first tier, serum is analyzed with a sensitive EIA or (less commonly) an indirect immunofluorescent antibody assay (IFA); specimens negative by the first-tier test need not be tested with a second-tier test (135). If the first-tier test is positive or equivocal, the serum sample is analyzed using standardized IgM and IgG immunoblots, and seropositivity is established only when one or both of the immunoblots is positive according to specific interpretive criteria (Table 1).

As an added measure to enhance specificity, interpretive guidelines state that a positive IgM immunoblot alone (in the absence of a positive IgG immunoblot) should not be used as evidence of active disease in patients with illness greater than 1 month's duration (135). Thus, a positive IgM response is meant primarily to support the diagnosis of Lyme borreliosis in patients with erythema migrans. The rationale for this caveat (sometimes termed the "1-month rule") was that most patients with untreated active infection for >1 month were expected to have a well-developed IgG antibody response, but with more experience it has been learned that it may take up to 2 months for an expanded IgG response to develop. However, IgM reactivity alone

lasting more than 1 to 2 months likely represents a false-positive result or indicates previous early Lyme borreliosis (136, 137).

(i) Performance characteristics. (a) *Clinical sensitivity in patients with erythema migrans.* In patients with untreated solitary erythema migrans lesions, the sensitivity of conventional two-tiered serologic testing is positively correlated with the duration of rash prior to serologic testing (114, 138). In a study by Wormser et al. (138), conventional two-tiered testing was 14% sensitive in patients with solitary erythema migrans lesions who were evaluated within 1 week after developing the rash, compared to 86% in patients with localized infection who were evaluated 22 to 30 days after symptom onset ($P < 0.001$). The duration of symptoms has less influence on sensitivity in patients presenting with multiple erythema migrans lesions, a clinical marker of disseminated infection, but at any given time point up to 3 weeks after onset of the rash, the sensitivity of two-tiered testing is higher in patients with multiple erythema migrans compared to those with solitary lesions (94, 138).

The clinical sensitivity of conventional two-tiered serologic testing in patients with erythema migrans is higher during convalescence (upon completion of 2 to 4 weeks of oral antimicrobial therapy) compared to acute-phase testing. In a large, prospective study of patients with culture-confirmed erythema migrans, Steere et al. reported that sensitivity increased to 53% in convalescence, compared to 17% at baseline, among patients with localized erythema migrans without evidence of dissemination (94). Among erythema migrans patients with evidence of dissemination, indicated either by multiple erythema migrans lesions and/or detection of *B. burgdorferi* DNA in peripheral blood by PCR, sensitivity in convalescence increased to 75%, compared to 43% prior to antibiotic therapy (94). Similar trends have been reported in several other studies (139, 140).

Besides the intrinsic delay in development of a detectable antibody response, the sensitivity of conventional two-tiered serologic testing in patients with erythema migrans is limited by the second-tier assay itself. Several studies have demonstrated that immunoblots are approximately half as sensitive compared to whole-cell sonicate EIAs, in the acute-phase of erythema migrans (138, 140, 141). Thus, when whole-cell sonicate EIAs are followed by immunoblots in a two-tiered testing algorithm, the overall performance is diminished by the insensitivity of immunoblots in early disease.

(b) *Clinical sensitivity in patients with early Lyme neuroborreliosis or Lyme carditis.* Data defining the clinical sensitivity of conventional two-tiered serologic testing are less robust for these clinical manifestations, compared to erythema migrans. Not only are these manifestations relatively uncommon, but direct detection methods capable of confirming a diagnosis of erythema migrans (142) less often yield positive results in Lyme neuroborreliosis or carditis and are of little use as reference methods against which to compare the performance of conventional two-tiered testing. The most convincing studies have defined cases of early Lyme neuroborreliosis or Lyme carditis based on objective clinical features (e.g., the presence of cranial nerve palsy, meningitis, peripheral neuropathy, radiculoneuropathy, or atrioventricular nodal block) and, if applicable, characteristic laboratory abnormalities (e.g., cerebrospinal fluid lymphocytic pleocytosis), and sometimes either concomitant or recent erythema migrans (94, 139–141, 143).

According to such studies, approximately 60 to 90% of patients with early Lyme neuroborreliosis or Lyme carditis are positive by conventional two-tiered serologic testing at initial presentation, prior to the administration of antimicrobial therapy (94, 139–141). However, clinical sensitivity would be higher (90 to 100%) if immunoblot interpretive guidelines did not restrict the use of IgM reactivity in the diagnosis of Lyme borreliosis to an illness of ≤ 1 month's duration (94, 139, 140). Some patients with these relatively early manifestations exhibit only IgM immunoblot seropositivity after 4 to 8 weeks of illness, whereas expansion of the IgG response is not yet robust enough to fulfill IgG immunoblot criteria—although some bands, primarily those corresponding to early antigens, are usually present on the IgG immunoblot (Fig. 2) (94, 139, 140).

TABLE 2 Infections and inflammatory conditions associated with falsely positive Lyme EIA results

Condition	Subcategory	Reference(s)
Infection	Spirochetal infections: syphilis, yaws, pinta, leptospirosis, relapsing fever,	143, 153, 175, 215,
	<i>Borrelia miyamotoi</i> infection	375, 376
	Tick-borne infections: anaplasmosis, RMSF	153, 233, 375
	Viral infections: Epstein-Barr virus, cytomegalovirus, varicella, parvovirus B19	143, 375, 377–379
Inflammatory disorders	Bacterial endocarditis	380
	Autoimmune: rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis	143, 215, 375, 380
	Periodontitis or ulcerative gingivitis	376
Pain syndromes	Fibromyalgia	239, 360, 381
Vaccination	Lymerix (OspA)	377

Thus, serologic assays (whole-cell sonicate EIAs and immunoblots) are highly sensitive in second-stage Lyme borreliosis, but the 1-month restriction degrades the calculated sensitivity of conventional two-tiered testing during this stage of the infection.

(c) *Clinical sensitivity in patients with Lyme arthritis.* In the northeastern U.S., almost all cases of late-stage Lyme borreliosis manifest with Lyme arthritis, usually developing months to as long as 2 years after initial infection. After *B. burgdorferi* infection of this duration, a strong IgG antibody response is detected in almost all patients (135). Therefore, the reported sensitivity of conventional two-tiered serologic testing among U.S. patients with Lyme arthritis is usually 100%, or only slightly less (94, 139–141, 144).

(d) *Specificity.* A major strength of conventional two-tiered serologic testing is its high specificity. When data from various control groups are combined, overall specificity is reported to be $\geq 99\%$ (94, 114, 139–141, 145). In the largest study to date, the conventional two-tiered algorithm's specificity among healthy blood donors ($n = 512$) who lived in regions of Lyme borreliosis nonendemicity was $\geq 99.8\%$ (95% confidence interval [CI], 98.9 to 100%) (141). Among asymptomatic control subjects living in regions of endemicity for Lyme borreliosis ($n = 1321$), specificity in the largest study was $\geq 98\%$ (95% CI, 98.8 to 99.7%) (141). The slightly lower specificity in comparison to control subjects from regions of nonendemicity likely reflects higher background seropositivity in individuals who have had past or asymptomatic infection. Among symptomatic subjects with no history of Lyme borreliosis but who have illnesses that can mimic Lyme borreliosis, or among subjects whose serum contains potentially interfering substances (Table 2), specificity is still $\geq 98\%$ (94, 114, 139–141, 145).

The high specificity of conventional two-tiered serologic testing is conferred primarily by the second-tier immunoblot, which is significantly more specific than the first-tier EIA (139–141). High immunoblot specificity is achieved by requiring evidence of multiple *B. burgdorferi*-specific antibodies (i.e., multiple specific bands) for a positive result. However, immunoblot specificity is also enhanced by testing only EIA-reactive samples; the specificity of immunoblots used as stand-alone tests is lower compared to conventional two-tiered testing (102, 146, 147). Also, the reported specificity of immunoblotting has usually been evaluated in the context of research investigations involving experienced laboratories. When immunoblots are performed by less experienced laboratories, weak bands are sometimes erroneously scored as “present,” when they should be ignored, resulting in falsely positive results. This is mostly a problem with IgM immunoblots (136, 137, 144), because the presence of only two specific bands is required for a positive result (Table 1). Incorrectly scoring just one or two weak IgM bands as “present” can change the outcome. In comparison, at least five specific bands must be present for a positive result in IgG immunoblotting.

The problems with visual interpretation of immunoblots can be partially overcome by the use of instrumentation to determine band intensity, but appropriate cutoff intensities have not been standardized or rigorously evaluated, and results must still be verified manually. Also, contrary to current interpretive guidelines (135), positive IgM immunoblots are sometimes inappropriately used as evidence of active disease in patients whose illness substantially exceeds 1 month's duration. In a retrospective

study of 182 patients referred to an infectious diseases subspecialist for presumptive Lyme borreliosis, 50 of 94 patients referred with a reportedly positive IgM Western blot (53%) were regarded as having a falsely positive IgM Western blot (137). Among those 50 patients, 45 (90%) had only a positive IgM Western blot (with a negative IgG Western blot) and symptoms >4 weeks duration at the time of testing; 39 (87%) had symptoms in excess of 8 weeks' duration (137).

(e) *Predictive value.* With any test, the predictive value of a positive or negative result depends on the sensitivity, specificity, and disease prevalence in the tested population, as follows:

$$\begin{aligned} \text{PPV} &= \frac{\text{sensitivity} \times \text{prevalence}}{(\text{sensitivity} \times \text{prevalence}) + [(1 - \text{specificity}) \times (1 - \text{prevalence})]} \\ \text{NPV} &= \frac{\text{specificity} \times (1 - \text{prevalence})}{[(1 - \text{sensitivity}) \times \text{prevalence}] + [\text{specificity} \times (1 - \text{prevalence})]} \end{aligned} \quad (1)$$

where PPV is the positive predictive value and NPV is the negative predictive value. Whereas sensitivity and specificity are fixed characteristics of the test itself, disease prevalence can vary greatly between geographical regions. This is certainly the case with tick-borne infections such as Lyme borreliosis, in which background disease prevalence is heavily influenced by the local ecology. Large regions of the United States are not endemic for Lyme borreliosis, and transmission within these regions is either extremely rare or nonexistent. Thus, despite the high specificity of conventional two-tiered serologic testing, its positive predictive value is low outside regions of endemicity. For example, in a study of patients in North Carolina, the positive predictive value of conventional two-tiered testing was only 10% among individuals who had not traveled to regions of endemicity (148). In nonendemic settings, positive serologic test results—even using highly specific assays and testing algorithms—are usually falsely positive and should be viewed with appropriate skepticism.

(ii) **First-tier assays.** (a) *First-generation enzyme immunoassays.* First-tier serodiagnostic tests for Lyme borreliosis may be manufactured using several different assay formats and target antigen preparations. The prototypical first-tier tests are “whole-cell sonicate” enzyme immunoassays (WCS EIAs). These assays are constructed by cultivating one or more *B. burgdorferi* strains in broth medium and preparing a protein lysate from the cultured spirochetes, for use in binding and detecting human anti-*B. burgdorferi* antibodies present in serum or plasma, typically using an indirect EIA format. In some EIA methods, a preabsorption procedure is incorporated during sample preparation, which may improve specificity by reducing cross-reactive antibodies (149–152), but it also reduces titers of homologous antibodies and thus decreases sensitivity (153–155). EIAs may be polyvalent, meaning capable of detecting human anti-*B. burgdorferi* antibodies of any isotype (i.e., “total” antibody detection) or they may be isotype-specific, usually targeting IgM-, IgG-, or IgA-class antibodies. IgA antibody responses are common in Lyme disease (156), including in neuroborreliosis (157). However, diagnostic criteria have been based solely on IgM and/or IgG responses, and it has not yet been determined whether measurement of IgA responses could have a beneficial role in diagnostics.

(b) *Second- and third-generation enzyme immunoassays.* First-generation EIAs, which are prepared from *B. burgdorferi* cellular lysates, contain innumerable different antigens and epitopes that bind human anti-*B. burgdorferi* antibodies, accounting (in part) for their higher sensitivity in comparison with immunoblotting. However, some immunodominant antigens (especially VlsE [100, 158–160]) are not expressed in appreciable quantity during *in vitro* *B. burgdorferi* culture and thus are absent or not well represented in assays prepared only from culture lysates. In addition, the use of a single *B. burgdorferi* isolate in first-tier assays (as is often done in the United States) may limit sensitivity, particularly for the detection of Lyme borreliosis acquired in Europe. In contrast with North America, several Bbsl species (*B. afzelii*, *B. garinii*, and *B. burgdorferi*)

are prevalent in Europe, and there is significant heterogeneity among them with respect to the antigens most relevant in serodiagnosis (38, 39).

To maximize sensitivity, second-generation EIAs were developed in which whole-cell lysates are supplemented with particular recombinant or purified antigens, often including multiple forms of the same antigen derived from locally prevalent Bbsl genospecies and strains. While such assays have been commonly used in Europe, few are available on the U.S. market, presumably owing to the comparative homogeneity among *B. burgdorferi* strains prevalent in North America.

One drawback of first- and second-generation EIAs is that inclusion of numerous antigens in the form of whole-cell protein lysates increases the likelihood of false-positive results from cross-reactive antibodies. Many infectious and inflammatory conditions can cause cross-reactivity in these assays (Table 2), and falsely positive reactions are also common among healthy controls subjects (140, 141, 143).

Recently, EIAs have been developed that contain only one or just a few antigens for binding host antibodies. These third-generation (or “next-generation”) EIAs are prepared using recombinant proteins, synthetic peptides, or synthetic chimeric proteins, representing immunodominant antigens or epitopes important in the antibody response to *B. burgdorferi* infection (92). This eliminates many potentially cross-reactive cellular proteins that do not induce a strong host antibody response (109, 161). Furthermore, by engineering short, synthetic peptide antigens or chimeric proteins, the least cross-reactive, best-conserved, and most immunodominant epitopes can be selected for inclusion. This strategy is intended to maximize specificity without sacrificing sensitivity (162). Finally, the use of recombinant or synthetic antigens greatly reduces between-lot variability during assay manufacturing, simplifying the process of producing consistent quality and performance. Assays based on whole-cell sonicates of cultured spirochetes may vary in their antigen composition from lot to lot, because some key antigens are differentially expressed depending on the growth phase at the time of harvest and on the number of serial passages to which the cultured strain is subjected (163–167).

(c) *VlsE*-based enzyme immunoassays. Several third-generation EIAs incorporate VlsE, or a portion of it, as an antigen target. VlsE (variable major protein [Vmp]-like sequence, expressed) is a 34- to 35-kDa surface lipoprotein that helps the spirochete evade the host immune response during infection by undergoing antigenic variation (168). The expressed portion of the *vlsE* gene contains six centrally located “variable regions” (VR-I through VR-VI) that recombine with multiple unexpressed cassette sequences located upstream on the same linear plasmid, resulting in a high degree of antigenic variation among *B. burgdorferi* clones infecting an individual host (159, 169).

The six variable regions of *vlsE* alternate with six conserved “invariable regions” (IR-I through IR-VI), and there are also invariable (but less well conserved) domains located at the 5′ and 3′ ends of the gene (159, 169, 170) (Fig. 4). During infection, VlsE stimulates a strong, early humoral immune response (168, 171), making it an attractive target for serodiagnostic assays. Interestingly, IgM-class antibodies do not bind well to VlsE epitopes and are less consistently detectable compared to IgG-class anti-VlsE antibodies, even at early time points; however, anti-VlsE IgG antibodies appear to develop very early during the course of infection, and IgM responses are usually not found in the absence of IgG reactivity (114, 161, 163). Thus, most single-antigen VlsE-based immunoassays are designed as polyvalent or IgG-specific assays, since there is little advantage in specifically targeting IgM-class antibodies.

Some third-generation assays use full-length, recombinant VlsE as an antigen target, whereas others use shorter peptide sequences corresponding to specific epitopes within the parent molecule. Among the latter, the best characterized is the C6 EIA, which employs a 25-mer oligopeptide (the “C6 peptide”) corresponding to the sixth invariable region (IR-VI) within VlsE. This peptide antigen has been a focus for serodiagnostic tests because, among the six invariant regions of VlsE, IR-VI is immunodominant and is the most conserved between strains and genospecies of Bbsl (101). Moreover,

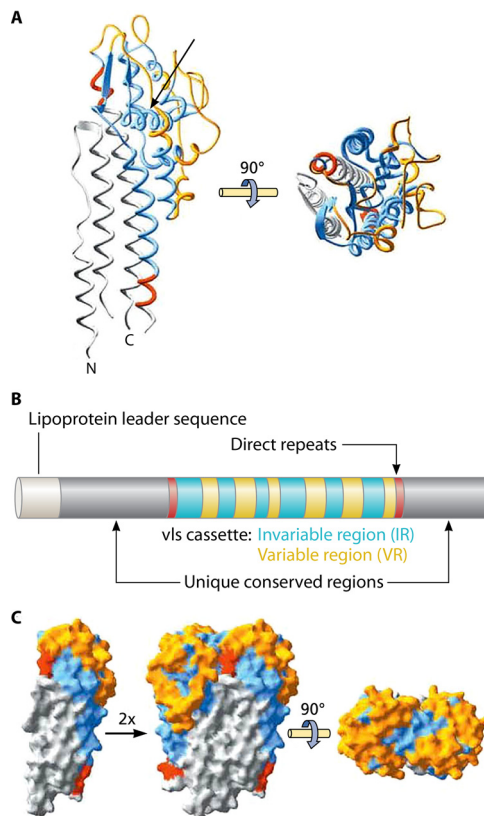


FIG 4 VlsE primary and tertiary structure. (A) The unique conserved N- and C-terminal regions are colored gray, direct repeats are red, and invariant regions of the cassette are blue, whereas variable cassette regions are orange. IR-VI (arrow) forms an alpha helix buried within the tertiary structure, with little surface exposure. (B) Schematic representation of the primary structure (color code as used in panel A). (C) Dimeric model of VlsE based on the crystal structure, illustrating how the formation of potential dimers could effectively shield invariant regions at the monomer-monomer interface. (Republished from reference 183 with permission of the American Society for Biochemistry and Molecular Biology. Note: The figure, as originally published, has been modified here with the addition of an arrow in panel A.)

anti-C6 antibodies are elicited during infection with all three RST genetic subtypes of *B. burgdorferi* (172). However, antigenic conservation between Bbsl genospecies is only partial (20, 173). In addition, reactivity in C6 EIAs is frequently seen in patients with *Borrelia miyamotoi* infection (174), as with WCS EIAs (175). Surprisingly, *B. miyamotoi*—a relapsing fever borrelia rather than a Lyme-related borrelia—is also a pathogen sometimes found in *I. scapularis* ticks.

Numerous publications have described laboratory-developed C6 EIAs (94, 114, 163). An FDA-cleared commercial C6 EIA was on the U.S. market for many years but is not currently available. In patients with early- or late-stage Lyme borreliosis, the previously available commercial C6 EIA was comparably sensitive to WCS EIAs, although some studies have found that it was less sensitive in first-stage Lyme borreliosis (acute erythema migrans) (140, 141, 176, 177) and others more (178, 179). However, the commercial C6 EIA's specificity was significantly greater compared to WCS EIAs in symptomatic or asymptomatic (healthy) control subjects (140, 141, 176, 180, 181).

Based on a limited number of head-to-head evaluations, serologic assays using full-length, recombinant VlsE (rVlsE) as the sole antigen target have similar sensitivity and specificity compared to C6 EIAs (114, 180, 182). However, assays employing full-length VlsE may not detect anti-C6 antibodies; more likely, they detect antibodies directed against other VlsE epitopes. When VlsE assumes its natural, tertiary structure, the IR-VI region (to which the C6 peptide corresponds) forms an α -helix that is buried within

the molecule and is largely inaccessible to antibodies (Fig. 4) (183). Thus, based on the resolved crystal structure of VlsE, anti-IR-VI antibodies should have minimal interaction with the intact parent molecule (183), and this has been demonstrated experimentally (184). Although studies to date indicate that C6 EIAs and rVlsE EIAs perform comparably, additional head-to-head evaluations are needed as it is possible that important differences in performance could be revealed among certain patient subpopulations.

(d) *Outer surface protein C-based enzyme immunoassays.* OspC is a surface lipoprotein that provokes a strong, early humoral immune response (98, 103, 185). Its immunodominance and usefulness in serodiagnostics have long been recognized; for example, OspC is one of the antigens scored when interpreting IgM or IgG Western blots according to CDC criteria established in 1995 (Table 1). The protein is encoded by a gene located on a circular plasmid (186). Unlike VlsE, which is not expressed until spirochetal transfer to a mammalian host, OspC is expressed while the spirochete is still contained within its tick vector during tick feeding (187). Thus, OspC is available to provoke a host immune response immediately upon initial infection, making it an attractive target for early antibody response detection in diagnostic assays.

Several EIAs have been developed using full-length recombinant OspC (rOspC) (21, 103, 118, 188–190). One complicating factor with this approach is the potential importance of matching the assay's OspC antigen with that expressed by locally circulating Bbsl strains. In regions like Europe where Bbsl strain diversity is great, there is substantial OspC antigenic diversity and the choice of OspC genotype for immunoassay development may affect performance (21, 39, 191–195). However, this problem has only minor practical significance, since strain-restricted OspC antibody responses are uncommon (196, 197). Of note, the IgM response to OspC wanes during prolonged infection (93, 197–199) and IgG class-switching to OspC is typically not detected in early disease (198).

As an alternative to rOspC, serologic assays have been made using a short (10-amino-acid) synthetic peptide called pepC10, which corresponds to a conserved motif located at the carboxy terminus of OspC (114, 198, 200). Whereas full-length rOspC is known to contain cross-reactive epitopes (21, 105, 198), the use of a single epitope (pepC10) improves specificity by eliminating these cross-reactive epitopes, although the effect is minor (198). Using a conserved epitope also helps address concerns about universality. However, these advantages are balanced by evidence that pepC10 is not among the immunodominant epitopes of OspC (201). In a head-to-head comparison between a rOspC IgM EIA and a pepC10 IgM EIA, the latter was slightly less sensitive in patients with EM (44% versus 36%, respectively; $P < 0.04$) when assay cutoffs were set to produce equal specificity between the two assays (198). A different OspC epitope, OspC1, was recently discovered through linear epitope mapping and may produce improved EIA sensitivity and specificity compared to pepC10 (105), although further study is needed.

(e) *Singleplex EIAs combining rVlsE or C6 peptide with rOspC or pepC10.* Multiantigen singleplex EIAs have been designed either by combining the OspC antigen (or pepC10) with VlsE (or C6) in a single assay, or running two single-antigen tests in parallel and combining results (*i.e.*, requiring reactivity in both assays for an overall interpretation of seropositive) (114, 146). Using this combination of antigens/epitopes, sensitivity can be improved in early Lyme borreliosis compared with either antigen alone, although specificity is slightly decreased (114, 144).

(f) *Other antigens and epitopes.* Although VlsE and OspC have been a focus of third-generation EIA development, assays based on many other antigens or epitopes have been developed or proposed. B-cell epitope mapping of specific *B. burgdorferi* protein antigens has accelerated this effort, helping to identify attractive peptide targets for assay development based on immunodominance, conservation across Bbsl genospecies and limited cross-reactivity (20, 105, 112, 113, 115, 202–204). Conversely, characterization of epitopes can show the nonutility of specific antigens due to extensive cross-reactivity in sera from control subjects (205). Epitope mapping has also shown

that humoral responses against distinct epitopes within a particular protein antigen may develop asynchronously, with potential implications for staging of the infection (171, 206). Broader proteomic approaches have revealed undiscovered antigens, and confirmed the immunogenicity of proteins already established as antigens, defining additional possible targets for assay development (108, 110). Rather than using a single epitope or antigen, many novel assays are being developed using a few individual epitopes or antigens together, either fused into chimeric molecules (162, 207, 208) or separated in multiplexed configurations (92, 146, 209–213).

(g) *Indirect immunofluorescent antibody assays.* EIAs have largely replaced IFAs as first-tier tests, although both are acceptable according to current guidelines (135). IFAs are typically manufactured by coating a multiwell microscope slide with whole *B. burgdorferi* spirochetes (214). After incubation with patient serum and staining, each slide well must be examined visually using a fluorescence microscope, a manual process which requires both skill and experience to avoid under- or overinterpretation. In contrast, the output of EIAs is measured objectively using a spectrophotometer or other instrumentation.

Several studies have demonstrated that EIAs are more specific and sensitive compared with IFAs (154, 215, 216). EIAs are also more convenient as first-tier assays, because in many cases they can be performed using semiautomated or fully automated instruments, enhancing throughput and allowing uni- or bidirectional interfaces with the laboratory information system. One advantage of IFAs, however, is that they are often designed as semiquantitative assays, producing an antibody titer; most commercial EIAs are qualitative tests. As with EIAs, IFAs may be designed as polyvalent assays or as isotype-specific assays.

(h) *Capture enzyme immunoassays.* Capture EIAs are formatted as sandwich ELISAs, and are usually immunoglobulin class-specific. Although substantially more complex than the indirect EIA method, the capture EIA method may be more sensitive in early Lyme borreliosis for the detection of a *B. burgdorferi* IgM antibody response (126). This assay design also lends itself to semiquantification but is more cumbersome than the indirect EIA format.

(i) *Quantifying the antibody response.* Although EIAs can be developed as qualitative, semiquantitative, or quantitative assays, most commercial *B. burgdorferi* EIAs are qualitative. Qualitative assays are interpreted categorically as positive, negative, or equivocal, whereas semiquantitative assays can determine relative antibody levels and may provide an antibody titer; quantitative assays provide the absolute antibody concentration. Although qualitative EIAs produce a numerical result—typically an optical density (OD) index value—this value is translated into a categorical result using predetermined cut-points, and the numerical value is not always reported in the medical record (depending on local preferences). OD index values can give a very rough idea of relative antibody levels (217), but this approach is imprecise. With a typical indirect EIA, the antibody concentration is related to OD by a logarithmic function (218) and when plotted on a logarithmic scale the dose-response curve is usually sigmoidal, with only the central region of the curve being linear.

Qualitative first-tier assays are usually adequate for testing individual samples, whereas semiquantitative or quantitative assays are particularly useful when comparing multiple serum samples collected from a single patient at different time points to assess for seroconversion, seroreversion, or reinfection. In some cases, these changes can be documented using qualitative assays, when serially collected samples transition from negative to positive or vice versa, but semiquantitative or quantitative assays are more informative since they reveal the amplitude of the antibody response at various time points. This is especially helpful when serially collected specimens are all reactive, but the antibody titer is rising or falling over time. Such changes cannot be assessed reliably using qualitative assays.

It has been suggested that first-tier EIA OD index values may be used to direct empirical antibiotic therapy in the absence of a second-tier assay result, if the value

exceeds a certain threshold. In two studies using qualitative Lyme borreliosis EIAs, Nigrovic and colleagues reported that high OD values are strongly predictive of true disease (217, 219). These authors concluded that sufficiently high EIA values might obviate supplemental immunoblotting or at least could inform clinical decision-making while waiting for supplemental test results.

(iii) Second-tier assays. (a) *Western blots.* Currently, most FDA-cleared second-tier serodiagnostic tests are immunoblots. Early second-tier immunoblots were prepared by Western blotting, and the Western blot test format is still commonly used (220). Although high-quality *B. burgdorferi* Western blot test strips are commercially available, visual interpretation of Western blots is complex and subjective. It involves locating bands of interest and then determining whether each band's intensity is sufficient to score it as present. In experienced hands, the method is reliable and reproducible (221), but in routine practice there is limited interlaboratory reproducibility (137). To reduce the subjectivity of visual interpretation, densitometric imaging techniques for measuring band intensity have been developed (102, 222, 223). These techniques are helpful, but standardized criteria for determining appropriate band intensity cutoffs using densitometry have not been established, and these interpretive aids are typically not FDA-cleared along with the test kits, meaning that imaging tools can be used as an aid but visual examination is still required to confirm preliminary results obtained in this manner.

(b) *Line immunoblots and microarray immunoblots.* A newer generation of second-tier serologic tests has become available. Rather than starting with a relatively crude protein lysate and transferring all the proteins (within a certain size range) onto a membrane, as in Western blotting, individual purified or recombinant antigens of interest are directly applied to the membrane in specified locations. The membrane can be configured to resemble a Western blot, with protein antigens applied in lines or bands across a narrow strip and sorted by molecular weight (223) (Fig. 2), in which case the assay may be called a "line immunoblot," "line immunoassay," or "ladder immunoblot." Initial IgM and IgG line immunoblot assays marketed in the United States have included the same 3 or 10 protein antigens, respectively, that are scored using standardized interpretive criteria (Table 1) (135).

Direct application of specific protein antigens to the membrane addresses an important drawback of Western blotting. Western blots prepared from culture lysates are frequently cluttered with uninformative bands that occur nearby those that are intended to be scored, because *B. burgdorferi* protein lysates contain numerous cross-reactive antigens that are common to, or similar to, antigens expressed by ubiquitous bacteria (155, 224). Antigen migration during electrophoresis of protein lysates can also vary from run to run or from one part of the gel to another, slightly altering band location from strip to strip. These features necessitate careful comparison of each test strip against a lot-specific band locator, to identify bands of interest—a subjective, time-intensive process that requires experience. Moreover, unrelated proteins of similar molecular weights can colocalize at an identical location on the strip, such that antibody cross-reactivity against uninformative protein antigens may produce bands that occur at the same location as those produced by reactivity against antigens of interest (110, 225). These potential pitfalls are avoided with line immunoblots, because only the antigens of interest are applied to the membrane, and the antigens are applied as bands that are well-separated from one another and evenly spaced across the test strip. This results in a less cluttered test strip that is easier to interpret.

The direct application of antigens to line immunoblots also allows more flexibility in optimizing their antigen composition. For example, OspC is an important early antigen in serodiagnostic testing but is not well conserved across Bbsl genospecies (105). Several commercial European line immunoblots therefore use a combination of OspC antigens derived from multiple genospecies in an attempt to improve sensitivity (226). Line immunoblots may also include antigens like VlsE that are not well represented in *B. burgdorferi* culture lysates (139, 226) (Fig. 2).

Although line immunoblots are formatted as membrane strips to resemble Western

blots, antibody detection assays using selected antigens or epitopes can be designed in other configurations (211, 212, 227). One FDA-cleared second-tier test retains the membrane surface of line immunoblots but reduces it to a compact circle that fits into wells of a standard 96-well microtiter plate (227). Recombinant or purified *B. burgdorferi* antigens—the same antigens that are scored in conventional Western blotting—are applied at defined positions creating IgM or IgG “microarray immunoblots” (227). The main advantage of this format, compared to Western or line immunoblots, may be that immunoblots can be processed using standard automated microtiter plate ELISA instruments, allowing for full automation without the need for a dedicated processing instrument, although after processing the microarrays must be interpreted using a special optical reader coupled with proprietary software (227). In one study, this microarray immunoblot assay demonstrated >95% agreement with Western immunoblot and line immunoblot assays, using a consensus standard, and reduced technologist time (227).

(iv) Interpretation of results. Conventional two-tiered testing can have three possible outcomes: (i) a negative first-tier test, in which case second-tier immunoblots should not be performed; (ii) a reactive (positive or equivocal) first-tier test, with negative IgM and IgG immunoblots; or (iii) a reactive first-tier test, with a positive IgM and/or IgG immunoblot. For each outcome, multiple potential interpretations exist. In an individual case, it is not always possible to determine which of the alternative interpretations is correct, but often the most likely possibility can be determined by correlating clinical features, patient history and risk factors with a detailed assessment of the laboratory findings (83, 84, 228).

(a) Negative first-tier test. A negative first-tier test may represent a true-negative result or a false-negative result, depending on the circumstances. In general, a negative first-tier test in a patient who has been ill for longer than a few weeks is evidence against *B. burgdorferi* infection, since most individuals with Lyme borreliosis will have mounted a detectable, specific antibody response by that time. In contrast, falsely negative results are frequent in patients who have been infected for only days (Fig. 1). If the patient will not be treated empirically, one could repeat the first-tier test after an additional several weeks to assess for delayed seroconversion (Fig. 5). If the patient has recently traveled and could have acquired Lyme borreliosis in a region outside North America, testing may be repeated using an assay capable of detecting antibodies directed against European species and strains, particularly *B. afzelii* and *B. garinii*. This is less of a problem if a U.S. assay with the C6 peptide antigen is used (18, 19). In an immunosuppressed patient, the antibody response may be less robust but is usually still detectable. Although rare, a person with profoundly deficient humoral immunity, such as a patient on chemotherapy targeting B cells (229–232), would not be able to mount an antibody response.

(b) Reactive first-tier test, with negative IgM and IgG immunoblots. Discordance between the first-tier and second-tier tests is frequent during early seroconversion in patients with erythema migrans or other early manifestations, because first-tier tests are typically more sensitive than immunoblots or similar second-tier assays. Similarly, a reactive (positive or equivocal) first-tier test with negative immunoblots or a negative first-tier test with a positive immunoblot is sometimes seen in patients with past, treated infection. In such cases, early antibiotic treatment may have blunted the humoral immune response, or an initial antibody response may have waned over time (partial seroreversion). Discordance between first- and second-tier tests should prompt additional workup if the probability of early Lyme borreliosis with partial seroconversion is high. In this scenario, antibody testing of paired serum samples collected at least 2 to 3 weeks apart may be useful.

First-tier tests are generally less specific than immunoblots, and false-positive first-tier tests with true-negative immunoblot results may be seen in patients with other illnesses, healthy individuals, or in those who received vaccination for Lyme disease with OspA (Table 2). In many cases, false reactivity in the first-tier test will produce an OD index measurement in the equivocal or low-positive range, but this is not a uniform

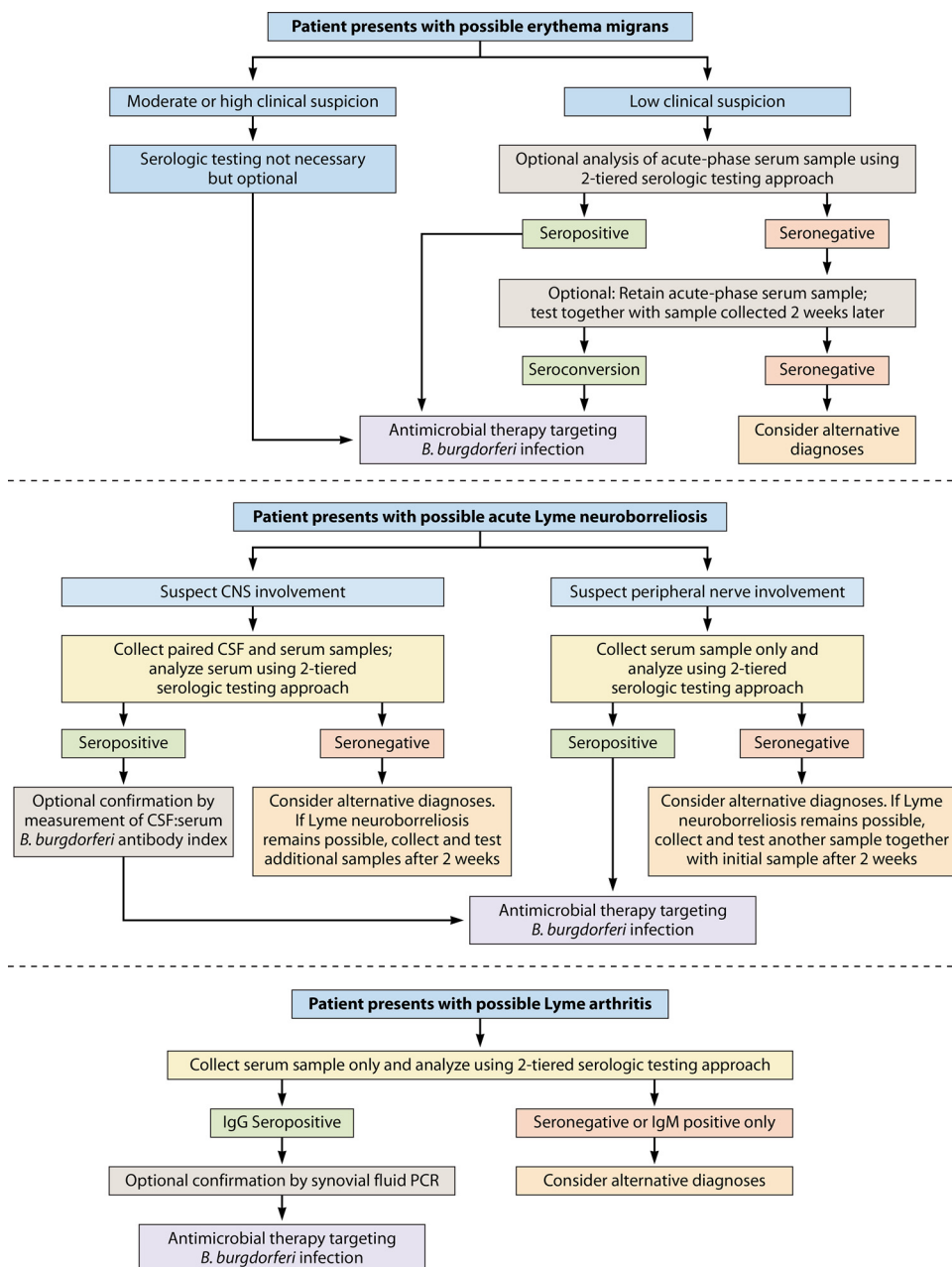


FIG 5 Recommended laboratory testing strategies for North American patients presenting with common manifestations of Lyme borreliosis.

finding (219). If the probability of active Lyme borreliosis is low, additional testing is not indicated when second-tier immunoblots are negative, especially if the duration of illness had exceeded 2 months at the time of sample collection.

(c) *Reactive first-tier test, with positive IgM and/or IgG immunoblots.* When a first-tier test is reactive and an IgM and/or IgG immunoblot is positive by accepted criteria (Table 1), the findings can support a diagnosis of Lyme borreliosis with a few caveats. It should be remembered that the clinical picture of active infection associated with IgM reactivity alone is usually erythema migrans. Also, because a detectable antibody response can persist for years despite successful eradication of *B. burgdorferi*, it is not possible based on standard serologic testing of a sample collected at a single time point to differentiate between active and past infection (122, 129, 130, 233). Clinical correlation is necessary, and testing of serially collected samples may help. An increase in antibody

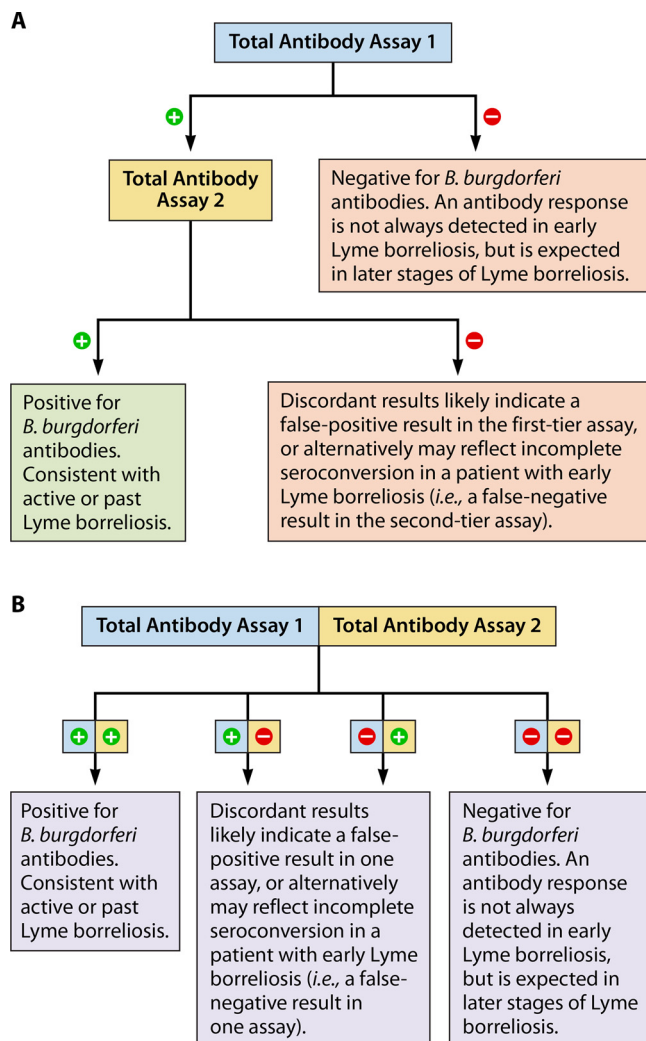


FIG 6 Modified two-tiered serologic testing protocols for the diagnosis of Lyme borreliosis, using two polyvalent (total antibody) assays. Two orthogonal EIAs are used, either sequentially (A) or concurrently (B).

titer with expansion of the antibody repertoire over time during the symptomatic period, and isotype switching from IgM to IgG antibodies supports the diagnosis of active *B. burgdorferi* infection. Conversely, demonstration of a contracting or waning antibody response is evidence of past (resolved) infection. Kinetic changes are best demonstrated using a semiquantitative or quantitative assay, if available. The immunoblot, a nonquantitative test, does not change much (or very slowly) after antibiotic therapy and resolution of infection (Fig. 2). Importantly, for valid comparisons of minor changes, it is necessary to analyze serially collected samples in the same assay run.

Although immunoblots are quite specific, false-positive immunoblot results do occur. Beyond detection of antibody reactivity related to past infection, other illnesses may produce falsely positive immunoblots. Cross-reactive antibodies may produce falsely positive first- and second-tier test results, since the tests are not independent (234). The problem is exacerbated by over-reading faint immunoblot bands and by the tendency of some laboratories to apply nonstandard interpretive criteria to Western blots (181). This disproportionately affects IgM blots (137), since only two specific bands are necessary to meet accepted criteria. Thus, nonstandard interpretive criteria should be avoided (181, 224).

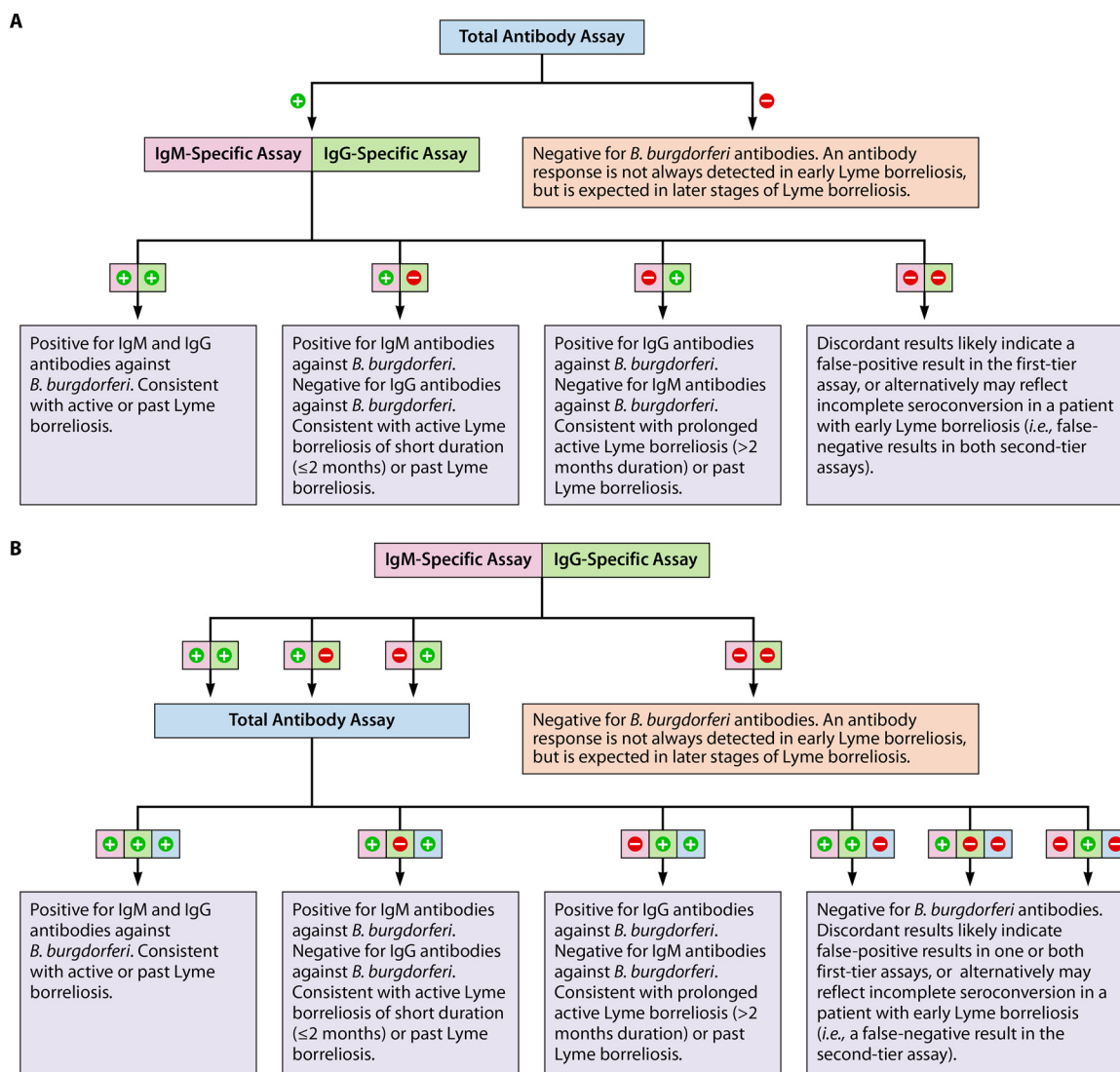


FIG 7 Modified two-tiered serologic testing for the diagnosis of Lyme borreliosis, using one polyvalent (total antibody) EIA, one IgM-specific EIA, and one IgG-specific EIA. The polyvalent assay can be used in the first tier, followed by orthogonal IgM- and IgG-specific assays in the second tier (panel A) or, alternatively, the IgM- and IgG-specific assays can be used in the first tier, followed by an orthogonal polyvalent assay (panel B). If desired, all three tests could be performed in parallel (concurrently) rather than sequentially (not shown).

Modified two-tiered serologic testing. Modified two-tiered testing (MTTT) for Lyme borreliosis involves the sequential or concurrent use of two or more orthogonal EIAs without the use of immunoblots (Fig. 6 and 7). As with conventional two-tiered testing, reactivity in two assays is required for a positive result in order to improve overall specificity. The CDC has endorsed the MTTT approach assuming the use of EIAs that have been cleared by the FDA for use in MTTT algorithms (235).

In the ideal situation, independent tests would be chosen for MTTT protocols to maximize overall specificity; by definition, independent tests are not susceptible to the same false-positive effects (236). Different EIAs for Lyme borreliosis are often not independent; however, pairing two nonindependent EIAs in an MTTT protocol may still improve specificity (237). Rather than independence, what is necessary is a sufficient degree of orthogonality between the tests, meaning that there are significant differences between the two tests in their format and design, and/or antigenic constituents (targets), that lead to a reduction in the probability of concurrent false-positive results

(238). With the advent of third-generation EIAs for Lyme borreliosis, which contain only one or just a few antigen targets, the opportunity has arisen to formulate MTTT protocols involving paired EIAs with substantially different antigenic constituents and thus a sufficient degree of orthogonality to improve specificity compared to either test alone.

Although two polyvalent (total antibody) tests can be paired, additional diagnostic information can be obtained by using separate IgM- and IgG-class specific EIAs in at least one tier of the MTTT approach (Fig. 7) (239). For example, the U.S. FDA has cleared a test system in which a polyvalent third-generation EIA is used in the first tier, followed by separate IgM and IgG WCS EIAs (240).

(i) Performance characteristics. One of the most validated MTTT protocols for Lyme borreliosis involves a WCS EIA (first-generation EIA) and a C6 EIA (third-generation EIA), performed sequentially or concurrently (18, 140, 141, 176, 177, 241, 242). Using this “2-EIA” protocol, specificity is greater than with either test alone and equal to the specificity of conventional two-tiered testing with a WCS EIA followed by immunoblots (92). This combination works well because the antigenic constituents of the two tests differ. The C6 peptide is either absent or minimally represented in WCS EIAs, since its parent molecule (VlsE) is not expressed well when *B. burgdorferi* is cultured *in vitro* (158). Furthermore, to the extent that the C6 parent molecule (VlsE) might be expressed *in vitro*, anti-C6 antibodies often do not react with VlsE and vice versa (184).

In patients with erythema migrans, MTTT is more sensitive compared to conventional two-tiered testing (92). This approach is also more sensitive in patients with early neuroborreliosis or acute Lyme carditis because of the “1-month rule,” after which IgM immunoblot reactivity is not used to support the diagnosis (92). In such patients, it is now known that it can take as long as 8 weeks for IgG antibodies to develop against enough antigens to meet IgG immunoblot criteria (140). The MTTT approach does not retain the 1-month rule, even if an IgM class-specific EIA is used in the algorithm (Fig. 7) and therefore patients who still have predominantly IgM reactivity during the 1- to 2-month period are nevertheless identified as seropositive. For example, if a polyvalent VlsE-based test is followed by separate IgM and IgG whole-cell sonicate tests, a reactive VlsE test and a positive IgM whole-cell test may be used to support the diagnosis of early Lyme disease even if symptoms have persisted for 2 months.

In modified two-tiered testing protocols, other third-generation assays can substitute for the C6 EIA, with equal sensitivity and specificity (176, 177, 239, 243). Similarly, two or more orthogonal third-generation EIAs can be used, without the use of a WCS EIA (177, 213, 239, 240, 243).

(ii) Advantages and limitations. Beyond improved sensitivity in early Lyme borreliosis, the modified two-tiered testing offers a number of advantages compared to conventional two-tiered testing, owing to elimination of the immunoblot component in the second tier (92, 244). Whereas blot interpretation is subjective, EIA results are obtained using an instrumented reader, which is an objective process. This should improve reproducibility between tests performed in different laboratories. In addition, protocols involving immunoblotting are more expensive than MTTT protocols (245).

The techniques involved in performing EIAs are also well within the capabilities of most clinical laboratories, even small ones. This is not the case with immunoblotting; due to the complexity of immunoblot interpretation and the limited availability of automated instrumentation to perform the testing procedures, the majority of clinical laboratories do not perform immunoblots on-site, requiring the use of reference laboratories. Thus, with MTTT, more clinical laboratories should be capable of performing both elements of two-tiered testing, further lowering costs and shortening turnaround time associated with reliance on commercial reference laboratories. If the EIAs are performed concurrently, instead of sequentially, even greater improvements in turnaround time can be achieved.

Finally, the results of MTTT are simpler to understand than immunoblot results. For example, patients with other diseases or healthy individuals commonly have a few bands on immunoblots, but do not meet criteria for a positive result. Banding patterns

that do not meet required criteria may cause confusion among physicians and patients. In contrast, for each tier of the MTTT algorithm, only a categorical result (positive, equivocal, or negative) is provided in the laboratory report, and the 1-month rule is eliminated. At least one test in each tier must be reactive (positive or equivocal) for an overall positive result. This should alleviate some of the confusion associated with serologic testing for Lyme borreliosis. For routine cases, in which objective signs consistent with a well-described Lyme borreliosis manifestation are present, with no history of Lyme borreliosis, a categorical result is likely sufficient.

The main limitation of MTTT is that, by eliminating the immunoblot component, detailed information about the host's antibody response to an array of individual antigens is lost (92, 244). This detailed information can provide insight into the extent and maturity of the antibody response, which is important in the evaluation of nonspecific, complex or rare manifestations of the disease. Examples may include patients with monoarticular arthritis; patients with an unusual manifestation of Lyme borreliosis, such as ocular involvement; patients without current or previous objective signs of Lyme borreliosis, such as erythema migrans (EM) or seventh nerve palsy, presenting with a possible manifestation of the disease; or patients who have been treated empirically for potential Lyme borreliosis but who have not responded.

A detailed analysis of the antibody response is also helpful in evaluating patients for potential *B. burgdorferi* reinfection, or for evaluating patients without a known history of Lyme borreliosis but who are living in areas of hyperendemicity, where the background prevalence of Lyme borreliosis is high (>5 to 10%) and the potential for baseline seroreactivity is commensurately elevated. Patients with symptomatic or asymptomatic past infection are likely to remain seropositive, which complicates the interpretation of serologic testing. In such cases, conventional two-tiered testing with immunoblotting may be more informative, especially if samples collected at multiple time points are analyzed. Alternatively, the immunoblot component can be replaced by a validated multiplexed serologic assay that is assembled on a more convenient platform.

Evaluation for *B. burgdorferi*-specific intrathecal antibody synthesis in patients with suspected CNS Lyme neuroborreliosis (i) Methodology. Because IgG antibodies diffuse back and forth across the blood-brain barrier, and because inflammatory conditions may disrupt the blood-brain barrier allowing diffusion of any immunoglobulin subtype, a test for intrathecal antibody synthesis must account for transudation of *B. burgdorferi*-specific antibodies across the blood-brain barrier from serum into cerebrospinal fluid (CSF). Thus, to evaluate for intrathecal antibody production, concomitant serum and CSF samples must be tested; analysis of CSF without analysis of paired serum is uninformative (246). The usual approach is to calculate the Lyme antibody index, which is the ratio of CSF *B. burgdorferi* antibody concentration to serum *B. burgdorferi* antibody concentration, corrected to account for total (polyclonal) immunoglobulin transudation across the blood-brain barrier (246). Although the antibody index can be measured using a polyvalent (total antibody) assay, the use of immunoglobulin class-specific immunoassays is preferred because the kinetics of passive diffusion differ based on the size of the immunoglobulin molecule, and this influences correction factors. Also, the immunoassays must be quantitative or semiquantitative. Immunoblots, which are not easily adapted as quantitative or semiquantitative assays, do not work as well as EIAs or IFAs for this application (247), and interpretive criteria have not been established for CSF analysis using immunoblots.

For IgG-class antibody determinations, a typical method—although methods currently are not standardized—is to measure the total IgG concentration in paired CSF and serum samples, usually by nephelometry, and calculate the CSF/serum total IgG ratio. In parallel, the concentration of *B. burgdorferi*-specific IgG antibody is measured in the CSF and serum samples, often using an indirect EIA technique adapted for semiquantitation. The CSF/serum *B. burgdorferi* IgG ratio is then divided by the CSF/serum total IgG ratio to determine the Lyme IgG antibody index (247), as follows:

$$\text{Lyme IgG antibody index} = \frac{[\text{CSF } B. burgdorferi \text{ IgG}] \times [\text{serum total IgG}]}{[\text{serum } B. burgdorferi \text{ IgG}] \times [\text{CSF total IgG}]} \quad (2)$$

By including the total IgG ratio in the calculation, passive diffusion of IgG antibody across the blood-brain barrier—whether at a normal rate or at an accelerated rate due to barrier dysfunction—is accounted for. An alternative approach is to dilute the serum sample to a predetermined final dilution suitable for the immunoassay in use, and then dilute the CSF sample such that its total IgG concentration matches that of the diluted serum sample (218). The diluted serum and CSF samples are then assayed for *B. burgdorferi* antibodies in the same run, and the Lyme IgG antibody index is determined by calculating the ratio of the CSF value to the serum value.

Normalizing the CSF/serum *B. burgdorferi* IgG ratio to the CSF/serum total IgG ratio (see equation 2) is a sound approach unless there is intrathecal synthesis of total (polyclonal) IgG, in addition to total IgG antibody present in the CSF due to transudation; in this case, the calculated Lyme antibody index is artificially low unless corrected. The presence or absence of intrathecal total IgG production can be assessed by comparing the CSF/serum total IgG ratio with the CSF/serum albumin ratio, as albumin is never produced intrathecally and can only enter the CSF by passive diffusion (247–249). If intrathecal total IgG production is determined to be present, the usual calculation (see equation 2) can be corrected using an equation involving the CSF/serum albumin ratio, as described by Reiber et al. (246, 249).

An alternative approach to determining the Lyme IgG antibody index is to normalize the CSF/serum *B. burgdorferi* IgG ratio by dividing it by the tetanus toxoid IgG ratio (247). This method avoids a minor pitfall of the method shown in equation 1, namely, that abundant production of intrathecal *B. burgdorferi*-specific IgG antibody increases the total CSF IgG concentration, paradoxically lowering the Lyme IgG antibody index value (247). In contrast, tetanus toxoid antibodies are not produced intrathecally and are produced independently from *B. burgdorferi* antibodies (247).

Instead of using correction factors, one can substitute a capture EIA technique for an indirect EIA technique. This practical method, which involves separate antibody class-specific assays (IgM, IgA, and IgG), inherently compares the amount of IgM, IgA, or IgG *B. burgdorferi*-specific antibody to the total amount of IgM, IgA, or IgG antibody, respectively, and thus obviates the need separately to measure total antibody in the samples (246). Semiquantitative values obtained by testing CSF samples are simply compared with values obtained using paired serum samples to obtain the Lyme antibody index (157, 250).

(ii) Results interpretation. If all *B. burgdorferi* antibody present in the CSF is due to transudation across the blood-brain barrier, the Lyme antibody index ratio for any immunoglobulin isotype should be 1.0. The higher the index ratio, the higher the likelihood that *B. burgdorferi*-specific antibody is being produced intrathecally (247). In our experience, we require an index of ≥ 1.3 before calling the test positive, although other cutoffs are used (249, 251), and a standard reference range has not been established. Although an elevated Lyme antibody index is evidence of intrathecal *B. burgdorferi* antibody production, it does not differentiate between active and past neuroborreliosis. As with serum antibody responses to *B. burgdorferi*, intrathecal antibody production can persist for months or years after treatment (252).

(iii) Clinical utility. Neuroborreliosis may involve the central nervous system (primarily the meninges), the peripheral nervous system, or both. In patients without apparent central nervous system involvement, and without a CSF lymphocytic or monocytic pleocytosis, production of *B. burgdorferi*-specific intrathecal antibodies is not expected (253), although it occurs occasionally (218, 251). In contrast, one study demonstrated that 12 of 13 United States patients with Lyme meningitis (92%) had an elevated Lyme antibody index, most commonly IgA but usually with antibodies to multiple isotypes (157). Another study demonstrated 53% sensitivity among 15 U.S. patients with Lyme meningoradiculitis and 100% specificity, using measurement of the Lyme total antibody index and an index value cutoff of 1.3; the sensitivity was 87%

and specificity was 93% when a cutoff of 0.9 was used (251). In this and a separate study (218), an elevated Lyme antibody index was also found in most patients with acute-onset or indolent Lyme encephalomyelitis, a rare entity. (Nearly all other studies of Lyme antibody index measurements have involved European patients.) Thus, evaluation of the Lyme antibody index is most useful in patients with potential CNS Lyme disease, especially acute Lyme meningitis—meaning those with exposure risk who have laboratory evidence of meningitis (CSF pleocytosis, with a lymphocyte or monocyte predominance) and detectable serum antibodies against *B. burgdorferi*. In U.S. patients, a serum antibody response is almost always detectable by the time signs or symptoms of neuroborreliosis have developed, at least with EIAs, and usually with immunoblots. Rarely, however, intrathecal antibody production of *B. burgdorferi*-specific antibodies precedes peripheral production. This phenomenon is well described in Europe (254) but has seldom been observed in North America (253).

Direct Detection Methods

Conventional or modified two-tiered serodiagnostic approaches have high sensitivity after the first several weeks of infection and are highly specific. Second- and third-generation tests have improved performance. However, this type of indirect test does not reliably distinguish active from past infection. In many infections, direct detection methods are available for that purpose, but such tests have been more problematic in Lyme borreliosis.

Direct microscopic visualization. In contrast with relapsing fever borreliae, direct visualization of Lyme-related borreliae in blood or other infected tissues is very challenging and easily subject to misinterpretation. The *in vivo* organism burden is usually so low in primary tissue samples that direct visual detection is not sensitive or practical as a first-line diagnostic approach, or even an adjunctive test (255). Using light microscopy, *B. burgdorferi* spirochetes can be visualized using Romanowsky stains (e.g., Giemsa) (11, 256) or with silver impregnation or immunohistochemical staining techniques (256). Gram staining is usually unsuccessful (256). Among silver impregnation staining methods, the preferred methods include the modified Dieterle silver stain (256, 257) or Bosma's modification of the Steiner stain (Bosma-Steiner) (256, 258). The Warthin-Starry stain will also highlight the organisms (256, 258, 259), but less sharply (258), and the routine (unmodified) Steiner stain does not work well (258). The spirochetes can be visualized using fluorescence microscopy, for example with the acridine orange stain (256) or with direct fluorescent antibody staining (260). Live, cultured organisms can be visualized using dark-field or phase-contrast microscopy (214).

In combination with serum antibody testing, microscopic tissue examination for *B. burgdorferi* spirochetes may serve an adjunctive diagnostic role, but direct examination requires considerable experience. Prior to antimicrobial treatment, Lyme-related borreliae can be visualized in skin samples from patients with erythema migrans (258, 261), occasionally in cardiac tissue from patients with Lyme carditis (262), or rarely in synovial tissue from patients with Lyme arthritis (258, 263). However, it is rare that one sees an entire organism, and it can be difficult to distinguish an organism from connective tissue fibers. Still, recognition of characteristic histopathological patterns can help support the diagnosis of erythema migrans or borrelial lymphocytoma, when the diagnosis is unclear, and is essential for the diagnosis of acrodermatitis chronica atrophicans (264–266). Direct microscopic examination of blood or cerebrospinal fluid is not useful in the diagnosis of Lyme borreliosis.

Borrelia culture. Lyme-related borreliae can be cultivated *in vivo* using laboratory animals (e.g., mice, hamsters, or rabbits) or *in vitro* using artificial media (214). Their nutritional requirements are substantial, and borreliae will not grow in routine bacteriologic culture media. Complex, highly enriched artificial medium is required for *in vitro* culture. The most successful recipes have been modifications of Kelly's medium, such as BSK-II medium (267), BSK-H medium (268), and Kelly-Pettenkofer (MKP) medium (269). Among these, BSK-H is the only commercially available formulation and is a more standardized medium, with less variability in its performance from batch to batch

and a longer shelf-life with refrigeration (268). Various antibiotics may be added to provide selectivity against commensal flora when skin or other nonsterile sites are cultured (214).

Lyme-related borreliae can be cultivated at core body temperature (37°C), but a slightly lower temperature incubation is optimal (32 to 34°C) (214). The spirochetes are microaerophilic; culture tubes should be filled to near capacity, to minimize ambient air in the container, and capped tightly to prevent loss of CO₂ from the medium (214). Although growth can frequently be detected within 1 or 2 weeks, 8 to 12 weeks of incubation should be allowed before termination (270). Growth detection may be performed using microscopy or PCR methods. Direct visualization of growing borreliae in culture, which is usually accomplished using dark-field microscopy or acridine orange staining, should be confirmed with PCR or a direct fluorescent antibody (DFA) staining method to improve specificity.

Although culture of erythema migrans skin lesions is the gold standard for research studies of early Lyme borreliosis, culture is rarely performed for diagnostic purposes. The need for expensive, complex media, technical expertise, and long incubation times makes this approach impractical for routine clinical use. The diagnostic yield of culture is also very low except in patients with erythema migrans, in whom moderate sensitivity may be obtained by culturing skin biopsy samples or blood. The sensitivity of skin biopsy culture in patients with untreated erythema migrans ranges from approximately 40 to 83% (142, 271–285). Skin biopsy samples should be taken from the leading edge (periphery) of the erythema migrans lesion (214) and must be obtained prior to initiation of antimicrobial therapy (283, 285).

Blood culture may be positive early in the infection, when erythema migrans is present, but is usually negative after that time. The yields are highest when plasma is cultured, as opposed to whole blood or serum (286), and when a high volume (at least 9 ml of plasma) is cultured (287). When a high-volume plasma culture is performed using microscopic examination for growth detection, the sensitivity in untreated patients with erythema migrans is approximately 40 to 50% (272, 286, 287). If culture growth detection is performed using PCR, the sensitivity can be increased to >70% (142, 288). A study reporting >90% sensitivity in incompletely characterized patients described as having Lyme borreliosis, using a novel blood culture method (289), was later invalidated (290, 291).

The sensitivity of plasma culture in untreated patients with extracutaneous objective manifestations of Lyme borreliosis is poor (<10%), unless there is concomitant erythema migrans. In a prospective study of U.S. patients with early Lyme neuroborreliosis, acute Lyme carditis, or Lyme-related arthritis or bursitis, the sensitivity of high-volume plasma culture with growth detection by microscopy was 6% (1 of 17, a patient with bilateral ankle bursitis of 21 days duration) among those without concomitant erythema migrans, compared to 44% (4 of 9) among patients with concomitant erythema migrans (292). All patients with a positive blood culture had been symptomatic for ≤30 days, and the authors of that study concluded that spirochetemia detectable by blood culture is mainly found in patients with a short duration of illness (292). Similarly, in a large European retrospective study, plasma cultures with growth detection by microscopy were positive in 1.9% of patients with borrelial lymphocytoma (1 of 53), 3.4% of patients with Lyme neuroborreliosis (6 of 176), 7.7% of patients with Lyme arthritis (1 of 13), and 1.5% of patients with acrodermatitis chronica atrophicans (3 of 200) (293). Among those with positive blood cultures, 36% (4 of 11) had concomitant erythema migrans. The median duration of symptoms prior to culture was 3.5 weeks, although the range was very wide (1 day to 2 years) (293). In a study of European patients with erythema migrans and concomitant signs and symptoms of nervous system involvement, the sensitivity of plasma culture was only 3% (4 of 127) prior to treatment (294). Another European study demonstrated 4% plasma culture sensitivity among patients with a clinical diagnosis of evident or suspected Lyme neuroborreliosis (295).

Culture of affected tissue sites in untreated patients with extracutaneous

manifestations of Lyme borreliosis is also a low-yield test. Four European studies reported CSF culture sensitivity of $\leq 10\%$ in patients with suspected Lyme neuroborreliosis (294–297), even though in one study, all patients had concomitant erythema migrans (294). Two studies involving culture of synovial fluid or tissue demonstrated 0% sensitivity in patients with untreated Lyme arthritis (298, 299). In contrast, nonviable spirochetes have occasionally been observed in synovial fluid (300, 301) and, as discussed below, PCR detection of *B. burgdorferi* nucleic acids in synovial fluid or tissue is often possible in Lyme arthritis. Thus, except in patients with erythema migrans, the low yield of culture even during active infection makes it an impractical test for diagnosis or to assess for cure at the conclusion of antimicrobial therapy.

Nucleic acid amplification tests. In diagnostic microbiology, when an organism cannot be cultivated or directly visualized using convenient methods, nucleic acid amplification tests (NAATs) have often been an excellent alternative. Assays targeting several different *B. burgdorferi* genes have been developed and evaluated, including chromosomal targets (e.g., *flaB* [302], *recA* [303], 16S rRNA gene [304], *oppA1* [9, 305]) and plasmid targets (e.g., *ospA* [306]). However, except for analysis of joint fluid prior to antimicrobial therapy in Lyme arthritis, PCR has been a low-yield procedure in Lyme borreliosis after the first weeks of infection. Also, NAATs do not work well as a test of cure. Bbsl nucleic acids may persist for weeks after antimicrobial therapy, and detection of nucleic acids (DNA or even mRNA) does not equate with the presence of viable spirochetes (271, 307–309).

(i) Blood PCR. In patients with solitary erythema migrans, the most common clinical manifestation of Lyme borreliosis, spirochetemia with *B. burgdorferi* cannot be reliably detected by whole blood or plasma PCR. Reported clinical sensitivity in patients with untreated solitary erythema migrans is often in the range of 30 to 50% (46, 142, 302, 310, 311) and sometimes lower (312, 313), likely reflecting differences in methodology.

The best opportunity to detect spirochetemia in patients with EM using blood PCR seems to be in those who present with features of very early dissemination, namely, those with multiple erythema migrans lesions and/or those with numerous constitutional symptoms. Among studies that compared plasma PCR sensitivity in patients with solitary EM versus patients with multiple EM lesions, one showed a significantly higher rate of positivity among the latter group (312), and two showed a nonsignificant trend in this direction (142, 302). Similarly, patients with EM and numerous systemic symptoms are more likely to have a positive plasma PCR result than those with few or no systemic symptoms (302, 312). However, even in this subset of patients with EM, plasma PCR is only approximately 60% sensitive or less (142, 302, 312). In studies that included patients with solitary and multiple erythema migrans but did not distinguish between them when calculating sensitivity or did not comment on whether erythema migrans lesions were single or multiple, the blood/plasma PCR positivity rate did not exceed 50% (46, 310, 311, 313), except in one study (62%) that used DNA extraction from large blood volumes plus target amplification prior to PCR (314).

Few studies have evaluated the clinical sensitivity of blood PCR in patients with noncutaneous manifestations of hematogenous dissemination. Two European studies reported sensitivity of 13 and 28% in patients with suspected Lyme neuroborreliosis, using plasma PCR assays (297, 315). Perhaps as an indication of the overall nonutility of blood PCR in the diagnosis of Lyme borreliosis and of the rampant overutilization of diagnostic testing when the pretest probability of infection is low, two retrospective studies conducted at a large Midwestern U.S. referral center reported blood PCR positivity rates of 0.1% (6 positive among 5,703 samples) and 0.4% (13 positive among 3,127 samples). Interestingly, in the latter study, 5 of the 13 positive samples contained *Borrelia mayonii* rather than *B. burgdorferi*, at copy numbers 50 to 8,000 times those of samples positive for *B. burgdorferi* during the same time period (9). These findings suggest that *B. mayonii* infection is more likely to present with detectable spirochetemia, and with a higher organism burden, although additional studies are needed. Currently,

B. mayonii is only known to occur in the upper Midwestern United States, and there is little information regarding the clinical manifestations of this infection.

(ii) **Solid-tissue or body fluid PCR.** PCR for *B. burgdorferi* can be performed on tissue or fluid samples, such as skin, CSF, or synovial fluid, depending on the suspected clinical manifestation. When applied to skin biopsy samples taken from erythema migrans lesions prior to antimicrobial therapy, the clinical sensitivity of various PCR assays has usually exceeded 50% (272, 274, 276, 278–281, 310, 316, 317) and sometimes reaches 70 to 80% (272, 274, 276, 278, 281, 316, 317). The wide range reflects differences in methodology, at least in part. For example, in one large study of 150 patients with erythema migrans, a PCR assay using primers targeting the flagellin gene, using agarose gel electrophoresis for amplicon detection, was 23% sensitive, while a nested PCR strategy targeting the intergenic *rrf-rrl* region (also with gel detection) was 71% sensitive when applied to the same skin samples (274). When directly compared to skin biopsy culture, PCR has usually provided superior sensitivity, although this is an inconsistent finding (142, 272, 274, 276, 278–281, 310, 316, 317). Most skin PCR methods yield superior sensitivity to that of conventional two-tiered serum antibody testing in patients with solitary erythema migrans, making skin PCR a reasonable alternative. However, many such patients present to primary care clinics or ambulatory clinics, where the necessary skin biopsy procedure may not be feasible. Furthermore, most clinical laboratories do not offer *B. burgdorferi* PCR on site, necessitating referral laboratory testing, which can be costly and may provide suboptimal turnaround time. Sample degradation during transport is also a risk. Thus, although PCR performed on skin samples of erythema migrans lesions provides good test performance in comparison to the reference method (antibody testing), there are practical impediments, and this approach is mainly used in research investigations.

B. burgdorferi PCR can be helpful clinically in cases of suspected Lyme arthritis. The sensitivity of PCR assays performed on synovial fluid or tissue prior to antimicrobial therapy exceeds 70% (271, 298, 318–321) in patients with Lyme arthritis. In contrast, the sensitivity of serum antibody testing approaches 100% (94, 114, 139, 141, 179). Therefore, serum antibody testing is the most appropriate first-line test in the diagnosis of Lyme arthritis (Fig. 5). However, in patients with possible past infection who are positive by two-tiered serum antibody testing, or in patients in whom one is considering a coexistent alternative diagnosis, a positive synovial fluid or tissue PCR result can improve diagnostic certainty, considering that seroreactivity can be detected for years in patients with past infection (122). On the other hand, PCR assays may remain positive for weeks or months after successful treatment for Lyme arthritis (271, 318, 321, 322). Therefore, a positive result does not prove active infection and, conversely, a negative result does not rule out active Lyme arthritis.

PCR for BbsI is very insensitive when performed on CSF collected from patients with suspected Lyme neuroborreliosis. Clinical sensitivity was only 5% in a U.S. study of patients with Lyme meningitis (323). In patients with acute neuroborreliosis, including (but not limited to) Lyme meningitis, the median sensitivity of CSF PCR was 21% according to a meta-analysis of published studies (324). As with blood PCR, poor clinical sensitivity combined with overutilization in patients with a low pretest probability of infection leads to enormous waste. In a large retrospective study at a major referral center, only 14 of 15,939 CSF PCR tests were positive (305). Considering that the sensitivity is very low, a negative CSF PCR test does not rule Lyme neuroborreliosis and contributes little to the evaluation of patients.

TEST SELECTION AND INTERPRETATION IN CLINICAL PRACTICE

Although diagnostic testing for Lyme borreliosis is not always clinically indicated during the first several weeks of infection, the first-line test, regardless of the clinical manifestation, is two-tiered serum antibody testing (either conventional, with EIA and immunoblotting, or modified, with two or more EIAs). However, the selection of

supplemental or alternative tests and the interpretation of test results are influenced by the clinical syndrome.

Erythema Migrans

When patients present with a slowly expanding, annular, erythematous skin lesion in the summer months, and there is established risk of Lyme borreliosis based on potential exposure to ixodid tick habitat in a region of endemicity, current guidelines recommend making a clinical diagnosis of erythema migrans rather than obtaining Lyme borreliosis diagnostic tests (84). This is because serologic testing is insensitive early in the infection and direct detection by culture or PCR requires a skin biopsy and specialized laboratory procedures. If serologic testing is pursued, there is a high risk of false-negative results, especially during the first week of infection. The risk of underdiagnosis stemming from falsely negative laboratory results is considered to outweigh the risk of overdiagnosis due to inaccurate clinical assessment, especially since treatment for erythema migrans usually works well and is relatively innocuous (84).

In some cases, patients may present with skin lesions that might be erythema migrans, but clinical suspicion is low. In such cases, diagnostic testing may be indicated, particularly if the patient will not be treated empirically for Lyme borreliosis. Paired serum samples should be obtained for serologic testing at the time of initial presentation and about 2 to 4 weeks later, since seroconversion may take several weeks (Fig. 5). However, if the acute-phase sample demonstrates seropositivity, the diagnosis of erythema migrans can be established and a convalescent-phase serum sample is not needed.

As adjunctive tests, *B. burgdorferi* culture or PCR testing of skin biopsy samples of EM skin lesions are often positive in true cases, and they provide direct, microbiologic proof of infection. However, many primary care providers are not credentialed to perform skin biopsies, necessitating referral to a dermatologist. Samples for high-volume plasma culture or blood PCR are not difficult to obtain (except in children), but these techniques are less sensitive than culture and PCR of skin biopsy samples in patients with EM. Moreover, culture of either skin biopsy specimens or plasma samples often requires incubation periods of several weeks in the laboratory before growth is detected, and *Borrelia* culture is not offered at most commercial reference laboratories. Thus, for practical reasons, patients with potential erythema migrans are usually treated on the basis of clinical features of the disease, but paired serum samples may be obtained for serologic analysis in an effort to support the diagnosis.

Early Lyme Neuroborreliosis

As with any evaluation for Lyme disease, it is important to assess whether the patient has had exposure to tick habitat in a region of endemicity. However, in contrast with erythema migrans, diagnostic testing is very important in the diagnosis of early Lyme neuroborreliosis (LNB) because signs and symptoms are insufficiently distinctive to allow for accurate clinical diagnosis. As with each manifestation of Lyme disease, the first-line test for LNB is two-tiered serologic testing, regardless of the anatomic site involved. As a supplemental test, measurement of the Lyme antibody index may be indicated if there is apparent central nervous system (CNS) involvement. Direct detection of *B. burgdorferi* spirochetes in CSF or blood, by PCR or culture, are low-yield procedures, so these tests are not routinely recommended.

Peripheral nervous system involvement. The most common manifestations of early LNB involving the PNS are cranial neuritis and radiculoneuritis. When these clinical findings are present without apparent CNS involvement, intrathecal antibody production is not expected, though both manifestations are occasionally accompanied by a CSF pleocytosis. Therefore, the diagnosis of early LNB usually relies upon demonstration of seroreactivity using two-tiered serologic testing. At the time of initial clinical presentation, before antimicrobial administration, reactivity in serum EIAs and IgM or IgG Western blots can be demonstrated in most patients (94, 114). In uncomplicated

cases, two or more EIAs (MTTT) may be preferable to EIA and immunoblotting (CTTT) due to higher sensitivity.

Central nervous system involvement. Lyme meningitis is the most common CNS manifestation of early LNB in the United States. Patients with Lyme meningitis are expected to have a CSF pleocytosis with lymphocyte predominance, and the CSF protein concentration is commonly elevated. When these laboratory abnormalities are present in a patient with meningitis who has had plausible recent exposure to ixodid tick habitat in a region of endemicity, the diagnosis of Lyme meningitis may be established using two-tiered serologic testing alone (Fig. 5). In U.S. patients, a serum antibody response is almost always detectable by the time signs or symptoms of LNB have developed. However, in patients with early Lyme neuroborreliosis who are seen within the first 8 weeks of infection, only the IgM test may be positive (Fig. 2).

Measurement of the Lyme antibody index may help confirm the diagnosis, as specificity is very high for LNB when the index is elevated. However, as with serum antibody testing, intrathecal antibody production may persist for months or years after treated LNB, so the Lyme antibody index cannot differentiate between active and past LNB (218, 325, 326). Analysis of paired CSF and serum samples is necessary; *B. burgdorferi* antibody testing of CSF alone, without concurrent serum analysis, is diagnostically uninformative. Demonstration of *B. burgdorferi*-specific antibodies in CSF, whether IgG, IgM, or IgA, does not alone indicate intrathecal production of *B. burgdorferi*-specific antibodies. There are no interpretive criteria for immunoblot testing of CSF.

The sensitivity of Lyme antibody index determination is slightly lower compared to two-tiered serologic testing, and therefore normal Lyme antibody indices do not rule out Lyme meningitis. Because serum antibody testing is more sensitive and usually positive in patients with Lyme meningitis, measurement of the Lyme antibody index is most useful in patients with detectable serum antibodies against *B. burgdorferi*. Rarely, however, intrathecal antibody production of *B. burgdorferi*-specific antibodies precedes peripheral production. This phenomenon is well described in Europe but has seldom been observed North America (253).

Lyme Arthritis

As with LNB, the clinical manifestations of Lyme arthritis are not pathognomonic, and the diagnosis must be supported by laboratory testing. Two-tiered serologic testing is the mainstay approach, since it is highly sensitive and specific. In the United States, Lyme arthritis is usually a late manifestation of the infection, and therefore an expanded IgG antibody response is expected, with at least 5 of 10 specific bands (and usually more) present on IgG immunoblots (Fig. 2). The absence of an expanded, specific IgG antibody response meeting current immunoblot interpretive criteria should raise substantial doubt about a potential diagnosis of Lyme arthritis or other late manifestations of Lyme borreliosis. Occasionally, especially in young children or European patients, Lyme arthritis can present earlier in the course of infection, with predominantly IgM reactivity and incomplete IgM-to-IgG class switching. In such cases, the IgM immunoblot may be positive, while the IgG Western blot has fewer than five specific bands and is negative by criteria (241). However, this phenomenon is rare in the United States, whereas it is more frequently reported in Europe, both in children and adults (18). Thus, in the United States, an IgM response alone is usually evidence against the diagnosis of Lyme arthritis. Western blots should not be performed on synovial fluid; the viscous, sticky nature of the fluid commonly gives false-positive results.

The main limitation of serologic testing in the diagnosis of Lyme arthritis is the inability to differentiate reliably between active from past infection. To confirm that seroreactivity is related to active arthritis in an untreated patient, PCR testing for *B. burgdorferi* DNA can be performed on synovial fluid as a supplemental test. Although it may still help with diagnosis, synovial fluid PCR is less informative once a patient has received antimicrobial therapy, as PCR remains positive for weeks to months after successful treatment, and therefore positive results do not equate with active infection.

Also, the sensitivity of synovial fluid PCR, although good, is significantly lower than that of serologic testing. Thus, a negative PCR result does not rule out Lyme arthritis, and synovial fluid PCR should only be pursued in seropositive patients.

Other direct detection methods, such as *B. burgdorferi* PCR of whole blood or plasma, or borrelia culture of synovial fluid or synovial tissue are much too insensitive in patients with Lyme arthritis to have any clinical utility. Also, while it is occasionally possible to detect *B. burgdorferi* spirochetes directly in synovial tissue using special staining techniques, the yield is low, the process is laborious, and it can be difficult to differentiate true spirochetes from artifact. Thus, direct microscopic detection for the routine diagnosis of Lyme arthritis is not a practical approach and is inferior to PCR as a supplement to serodiagnostic testing.

After successful treatment, the amount of antibody to *B. burgdorferi* declines gradually, as determined by EIA, but to obtain accurate results, serial samples need to be tested using the same quantitative or semiquantitative assay in the same run. Since commercial laboratories rarely save samples, repeat antibody testing has not been recommended. Immunoblots, which are nonquantitative tests, do not change much (or very slowly) after antibiotic therapy and are therefore not useful tests in assessing the response to antibiotic therapy.

OUTLOOKS

Insights recently gained through investigating the basic biology of Lyme-related borreliae, and the immune and inflammatory responses they elicit in the human host, are now being leveraged in attempts to improve diagnostic tests. The topics below address areas of active research, in which promising initial results have been reported but comprehensive validation is needed to assess analytical performance, clinical validity, and practical feasibility.

Indirect Detection Methods

Rapid, point-of-care serologic testing. Currently, serologic tests for Lyme borreliosis are typically performed in central hospital laboratories or at commercial referral laboratories. Either way, this approach involves substantial delay in producing test results. Although subject to the same limitations as other serologic tests, an accurate, rapid assay that could be performed and interpreted near the point-of-care, during a patient visit, would be valuable in evaluating patients with suspected early Lyme neuroborreliosis, Lyme carditis (270), or Lyme arthritis, because a detectable antibody response is expected in most true cases and diagnosis is difficult to establish based on clinical features alone. Furthermore, emergency ward or urgent care providers must promptly make consequential decisions that would be better informed by a rapid serologic test. For example, physicians evaluating patients with facial nerve palsy must decide whether to provide antimicrobials directed against the agent of Lyme borreliosis or corticosteroids to treat Bell's palsy. In children with new-onset arthritis affecting one or more large joints, physicians must determine whether to favor septic arthritis, in which case an urgent, operative joint washout may be indicated, along with antimicrobial therapy, or whether to favor Lyme arthritis, in which case a washout would be unnecessary.

Although rapid serologic assays in lateral flow format have been developed and marketed, so far they have been intended as first-tier tests in a two-tiered testing protocol, requiring second-tier immunoblots when the rapid assay is positive (327). The need for second-tier testing undermines the potential advantage of rapid assays. Thus, the availability of a stand-alone rapid assay will be necessary before rapid serologic tests can have much of an impact.

Stand-alone serologic tests. As with rapid assays, all commercially available EIAs are intended to be used in two-tiered testing protocols, rather than as stand-alone tests. This is primarily because none has achieved equal specificity compared with two-tiered testing (92). The need to perform two separate tests before establishing seropositivity generally increases cost and extends turnaround time, especially when

immunoblots are used as second-tier tests (245). Recent advances in assay development, especially the advent of assays using synthetic peptides, chimeric proteins or recombinant protein targets selected for minimal cross-reactivity, are likely to result in stand-alone serologic assays that match or exceed the performance (sensitivity and specificity) of two-tiered testing (92, 212).

Multiplexed serologic tests. Unlike EIAs, Western blots and the newer line or microarray immunoblots are intended to provide detailed information about the humoral immune response, indicating the presence or absence of reactivity against multiple distinct antigen targets. Beyond the use of immunoblots, alternative solid-phase surfaces can substitute for the membrane, for example by the application of antigens to bead particles that are analyzed by flow cytometry (146, 209, 210) or using a microfluidics-based “lab on a chip” (212, 328). These alternative platforms are highly flexible and may facilitate efforts to reimagine the antigenic constituents of multiplexed serologic assays. Although it remains informative to assess antibody reactivity against the three antigens currently scored in IgM immunoblotting and the ten antigens currently scored in IgG immunoblotting, it is now recognized that some of these antigens are highly cross-reactive and lack specificity for *B. burgdorferi* infection (155, 205). An optimal multiplexed assay might retain some of the antigens currently scored on immunoblots but replace cross-reactive antigens with more informative antigens or epitopes that were not recognized at the time that current immunoblot interpretive criteria were developed (112, 113, 209, 329) and/or are not well expressed in cultured spirochetes (e.g., VlsE, DbpA, or BBK32) (325). One analysis indicated that more than 5 and possibly as many as 25 different antigens or epitopes may be needed in a multiplexed assay for optimal discrimination between Lyme borreliosis cases and control subjects (108). Such an assay could eventually replace immunoblotting as a second-tier test in a two-tiered testing protocol, or conceivably could work as a stand-alone assay, assuming sufficient clinical specificity (92).

Antibody avidity tests. An important limitation of serologic testing is the difficulty in distinguishing antibody reactivity due to past (remote) infection from reactivity caused by active or recent infection. IgG avidity assays have been used to make this distinction when standard serologic assays have detected antibodies to cytomegalovirus (CMV), *Toxoplasma*, or rubella virus, and this approach has also been proposed for Lyme borreliosis. The principle of avidity testing is that specific IgG antibodies become more avid (i.e., bind their target with greater affinity) as the antibody response matures over time. Individuals with recently acquired CMV, toxoplasmosis, or rubella virus infection are expected to have lower IgG avidity compared to those whose infection was remote. However, a major difference is that reinfection is uncommon with these infections but is not uncommon after early Lyme borreliosis, likely complicating interpretation of antibody avidity tests. Furthermore, most patients with Lyme disease are treated at an early stage, and it is not known whether patients with previous (remote) early infection have high- or low-avidity *B. burgdorferi* IgG antibodies. Initial studies have not addressed these limitations (330–332), and a detailed understanding of changes in antibody avidity during the course of natural infection is lacking. Therefore, the potential utility of IgG avidity testing remains to be determined in Lyme borreliosis.

Gamma interferon release assays. Studies using *ex-vivo* T-cell proliferation assays have demonstrated that *B. burgdorferi* antigens stimulate T-cell activation in patients with Lyme borreliosis (333, 334), but this proliferative response is fairly nonspecific (335). T-cell activation in Lyme borreliosis also leads to production of proinflammatory cytokines, especially interferon gamma, as measured using gamma interferon release assays (IGRAs) (336–339). In some cases, strong interferon gamma responses can be observed shortly after initial infection (336, 340, 341), and it is possible that IGRAs could be capable of detecting the infection earlier than antibody tests during the serologic window period. In addition, a recent study used an IGRA to demonstrate significant reductions in gamma interferon release after antimicrobial therapy in patients with erythema migrans (341). These findings suggest a potential use for IGRAs in

differentiating between active infection and past (successfully treated) infection, although the authors of a separate study reached the opposite conclusion using a different IGRA (342) and further study is needed.

CXCL13. In the setting of neuroinflammation, a chemokine called CXCL13 is upregulated by monocytes/macrophages and dendritic cells, attracting B cells to the central nervous system to promote a local humoral immune response (343–346). In patients with acute Lyme meningitis, there is strong correlation between elevated CSF CXCL13 levels and elevated Lyme antibody indices (295, 347–350), and CXCL13 elevation may develop earlier during the course of infection (346, 351–353). Thus, quantitative measurement of CSF CXCL13 concentrations using simple ELISAs has been suggested as a sensitive and convenient diagnostic tool. CXCL13 levels appear to decline rapidly following antimicrobial therapy (347, 348, 351, 352, 354), potentially making this biomarker more suitable for monitoring response to therapy than measurement of the intrathecal antibody response, which can persist for months to years (252, 354).

Importantly, however, an elevated CSF CXCL13 concentration is associated with many other medical conditions, including meningitis caused by other infectious agents, HIV infection, neurosyphilis, CNS lymphoma, and multiple sclerosis (347–351, 354–356). There is also overlap between CSF CXCL13 levels measured in patients with definite or possible Lyme neuroborreliosis and levels measured in patients with many of these other infectious or inflammatory conditions (350). Thus, it is not yet clear whether CXCL13 elevations due to Lyme meningitis can be differentiated from other causes based on determining CXCL13 concentration or other measurements. Moreover, methods and reference ranges have not been standardized. These are significant challenges, and it remains to be determined whether chemokine measurement will have a place in clinical diagnostics for Lyme borreliosis.

Metabolomics and proteomics. Molins et al. recently applied an unbiased, iterative machine-learning strategy to discover signature metabolic biomarkers of early Lyme borreliosis (357). In this approach, a discovery set of serum samples from patients with untreated erythema migrans or healthy control subjects was analyzed by liquid chromatography-mass spectrometry for small-molecule metabolites. The data were used to develop and refine a metabolomics biosignature that could differentiate patients with early Lyme borreliosis (erythema migrans) from control subjects, based on shifts in the abundance of selected metabolites in one group compared to the other (357). When challenged with blinded samples, the biosignature achieved 88% sensitivity (95% CI, 84 to 95%) and 95% specificity (95% CI, 90 to 100%) in classifying cases and controls (357). Remarkably, this model could correctly classify most seronegative cases of confirmed early Lyme borreliosis, demonstrating its potential to reduce the serologic window period (357). Several biosignature constituents were known inflammatory mediators or markers (357), supporting other reports indicating that elements of the inflammatory response may be detectably altered in advance of seroconversion (358). Follow-up studies demonstrated that serum metabolic profiling could differentiate cases of EM from STARI, a Lone Star tick-associated illness that produces EM-like skin lesions (359), and that metabolic biosignatures of early Lyme disease are also identifiable in urine samples (360).

Another group reported on the use of proteomics rather than metabolomics in developing diagnostics for Lyme borreliosis (361). In this approach, targeted mass spectrometry was used to measure particular serum proteins of interest, which included acute-phase proteins involved in the innate immune response and proteins present only in specific organ systems that are frequently affected in Lyme borreliosis. The study identified 10 proteins whose serum levels were significantly altered in patients with acute erythema migrans compared to healthy control subjects. The sensitivity of the method in patients with erythema migrans was 75%, but the specificity was only 90% (361).

Although the experimental techniques used in proteomic and metabolomic studies are likely too complex and costly to be practical as routine diagnostic tests, it is

possible that further studies will define and validate a manageable list of key diagnostic biomarkers, the measurement of which could be feasible on a large scale. Studies on the value of metabolomics or proteomics in later stages of Lyme borreliosis are also needed.

Direct Detection Methods

Nucleic acid amplification tests. Nucleic acid amplification tests (NAATs) involving standard PCR or real-time PCR methods have been insensitive for the diagnosis of early or late Lyme borreliosis when applied to readily obtainable body fluid samples such as blood or CSF (228, 305, 362). To some extent, this may be a function of the infection's pathophysiology, although this remains largely speculative. For example, in some cases of localized EM, it is possible that the infection is entirely confined to the skin, without any associated spirochetemia even at very low concentration. When there is involvement of the nervous system, heart or joints, spirochetemia must occur in order to seed these areas, but it is possible that it is only transient or intermittent and can only be detected if the timing of specimen collection is fortuitous. When there is central nervous system involvement, it is possible that spirochetes do not always enter the CSF or may not be present continuously. If any of these suppositions is true, then direct detection of *B. burgdorferi* in blood or CSF using NAATs will not be possible in a subset of cases no matter how low the assay's limit of detection may be. On the other hand, the reported insensitivity of blood and CSF NAATs could in part be related to inadequate analytical sensitivity, and thus it is possible that method enhancements could improve performance.

Numerous variables can affect the analytical sensitivity of PCR assays, including the starting sample volume, the sample type (whole blood, plasma, serum, CSF, etc.), the extraction method, the amplification target and primer sequences, and the efficiency of the amplification reaction (which in turn is influenced by PCR chemistry and cycling conditions) (363). These variables have not been systematically evaluated in Lyme borreliosis to determine the optimal assay design. Nevertheless, a sufficient number of assay designs have been individually evaluated to suggest that a major improvement in analytical sensitivity is unlikely to arise from choosing novel PCR targets, further optimizing PCR chemistry, or combining multiple different PCR assays for use on a single sample. Rather, modifications to pre-PCR steps, such as increasing the volume of sample processed (302, 309, 311, 314) or amplifying *B. burgdorferi* DNA present in the sample prior to running the PCR test (314, 364), are more likely to have a significant impact on performance (362). It is also possible that new PCR technologies better equipped to detect low-abundance targets, such as digital PCR (365), will prove important.

Metagenomics. A potential alternative to NAATs is detection of *B. burgdorferi* genomic DNA in plasma or CSF samples, using shotgun metagenomics. This method involves unbiased ("shotgun") deep sequencing applied to primary samples, with the aim of detecting any microbial DNA present (366). It has been validated as a clinical diagnostic tool capable of detecting circulating fragments of genomic DNA from a wide variety of bacteria, fungi, viruses, and parasites in peripheral blood (plasma) (367, 368), and its utility in detecting *B. burgdorferi* infection was recently established (240).

An alternative to shotgun metagenomics is targeted deep sequencing, in which the sample is enriched for genomic DNA of a targeted pathogen using a NAAT or a hybrid capture technique prior to deep sequencing (362). Speculatively, this approach might be more sensitive compared than unbiased sequencing and would simplify data analysis to some extent. Either approach could also provide information about the species and strain type of the infecting agent (362).

Antigen detection. As with nucleic acid detection, infection with various agents can sometimes be demonstrated by detection of signature microbial proteins or carbohydrates in body fluids such as urine, serum, or CSF. Although *B. burgdorferi* antigen detection methods have been reported (369–372) and are even available for clinical diagnostic use at some commercial reference laboratories, independent confirmation

of reported study findings or validation of proposed assays is lacking (373). Also, the reported methods all involve detection of *B. burgdorferi* OspA in human body fluids, which is biologically implausible since OspA expression is suppressed during early human infection (119, 325). Nevertheless, advances in our understanding of the *B. burgdorferi* proteome (374) and in antigen concentration/enrichment methods (362) have prompted continued interest in antigen detection as a potential diagnostic tool for Lyme borreliosis.

Outlook Summary

As high-performing next-generation serologic assays and modified two-tiered testing algorithms become more widely adopted, the false-negative window period of serologic testing will be narrowed to its physiological limit. The remaining window, unavoidable due to the kinetics of the antibody response, will need to be closed either with improved direct diagnostic tests or with non-antibody-based indirect tests, such as cytokine release assays or metabolite-based assays. Direct detection tests involving serum or urine would be highly preferred, but components of the infecting spirochete (nucleic acids, protein antigens, etc.) may not be consistently present even in early infection and therefore may not be reliably detectable in those sample types even using the most sensitive methods. If not, indirect tests may be developed that can assist, but in general these are less likely than direct tests to provide clear-cut answers, particularly because indirect tests of any type rely on detecting responses in the host that take time to develop, even if the window is narrower compared to serology. Thus, the pathophysiology and kinetics of the infection may limit the ability to completely close the window period.

The other major problem in Lyme borreliosis diagnostics is the difficulty of differentiating active from past infection, especially when serologic test results from only a single time point are available. The solution will likely involve either improved direct detection or non-antibody-based indirect tests. It is possible that increasingly sensitive direct detection methods will be able to detect spirochetal components more consistently than current tests, but the limiting factor is whether such components are reliably present in readily obtainable fluids, particularly in later stages of the infection. Non-antibody indirect markers of infection may be developed, but identifying discriminatory cutoffs may prove difficult. Much research is now in progress to address these limitations (92, 362), which is likely to lead to further improvements in diagnostic testing for Lyme borreliosis.

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John A. Branda, M.D., received his M.D. degree from Harvard Medical School in 2000. He completed residency training in Anatomic and Clinical Pathology at Massachusetts General Hospital (MGH), followed by a fellowship at MGH in Clinical Microbiology. He joined the MGH Pathology faculty in 2004 and is currently an Associate Pathologist and Associate Director of the Clinical Microbiology Laboratories. He also serves as Associate Professor of Pathology at Harvard Medical School. His research interests include improving patient care through the development of new diagnostic testing strategies and through utilization management of laboratory testing and antimicrobial agents. He has a special interest in diagnostic testing for tick-borne illnesses, such as borreliosis, babesiosis, anaplasmosis, and tularemia.



Allen C. Steere, M.D., serves as Professor of Medicine at Harvard Medical School and Director of Translational Research in Rheumatology at Massachusetts General Hospital. His research career has been centered on the elucidation of Lyme disease. Forty-five years ago, he led the team that evaluated a cluster of children with arthritis in Lyme, CT, which led to the discovery of Lyme disease. He has subsequently studied the clinical manifestations, epidemiology, pathogenesis, diagnosis, treatment, and prevention of this complex infection. During the 1990s, he was principal investigator of the SmithKline Beecham Lyme disease vaccine trial, which led to licensing of the first vaccine for Lyme disease. His current research is focused on infection-induced immune responses that stimulate autoimmune responses in various forms of chronic inflammatory arthritis, including postinfectious Lyme arthritis.

